ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
FISCAL YEAR 1975

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
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DIRECTOR

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ANNUAL REPORT OF PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
JULY 1, 1974 THROUGH JUNE 30, 1975

The National Institute of Allergy and Infectious Diseases conducts and supports a broad program of research directed at improved ways of diagnosing, treating, and preventing allergic conditions and infectious diseases.

Institute programs have continued, in general, last year's pattern. Some disease areas have received increased support through extramural grants, contracts, and the intramural area. These include hepatitis, influenza vaccine development and studies on sexually transmitted diseases. Research on asthma and other allergic diseases continues to form a major segment of the Institute program stressing the acquisition of scientific knowledge for application of diagnostic, preventive and therapeutic measures.

Changes in the support of research training with the emphasis on fellowships has been satisfactorily accomplished though leading to a great deal of uncertainty for recipients during this year. Future activities in training will depend upon legislation now before the Congress. Procedures for the assurance for protection of human subjects have been put in practice successfully and are well accepted by grantees.

New requirements under the Freedom of Information Act have made it necessary to provide detailed information on funded grants to organizations having special concern for the conduct of clinical research. These requests appear to have been handled satisfactorily for the moment though requiring an unexpected amount of staff time and Institute expense.

The Office of Research Reporting and Public Response (ORRPR) has prepared approximately 35 "Research Reports" during the year. These non-technical summaries of significant research results published in the scientific literature by NIAID scientists, grantees, and contractors provide a useful base for more lengthy Institute reports prepared for both internal and external distribution. Individual "Research Reports" are sent to NIAID senior staff, to members of NIAID advisory groups, and to science writers on request.

ORRPR has also strengthened the Institute's efforts in communicating with the general public and with practicing allergists through development of its allergy publication program.

The Institute's series of pamphlets on allergies, initiated last year, resulted in a Blue Pencil Award for excellence from the Federal Editors' Association. Two new pamphlets in the series, "Drug Allergy" and "Mold Allergy", were produced this year, and the popularity of the first three pamphlets in the series required our going "back to press" on all three.

The ORRPR received, as well, a Blue Pencil Award for "Hepatitis", produced in FY 1974.

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For several years, we have been distributing two publications -- "Bacteria, The Littlest Cells", and "Viruses, On the Border of Life" -- to interest high school students in science and possible careers in medicine and the health sciences. This year, ORRPR produced a third such booklet, "Miscellaneous Microbes, The Nonconformists". This new publication discusses some of the specialized forms of bacteria -- including rickettsia, chlamydia, mycoplasma and the actinomycetes -- not covered in the other bacteria booklet.

We are now distributing 21 infectious disease research fact sheets. Seven of these fact sheets were updated this year, and a new one, "Strep Infections" includes discussions of rheumatic fever and glomerulonephritis. For the first time, ORRPR cooperated with the Consumer Products Information Center, GSA and supplied 25,000 copies of Mononucleosis for national distribution from the Center's Pueblo, Colorado, operation. The volume of ORRPR publications distributed has steadily increased -- from approximately 18,000 in FY 1972 to more than 135,000 this year.

The ORRPR helped develop an exhibit for the Alumni Reunion and the NIH Open House depicting the Institute's current intramural research activities and highlights of past accomplishments. The exhibit is tied in with NIH's observance of the Nation's Bicentennial celebration. A handout, "Research Programs of NIAID", briefly describes the Institute's research efforts. The ORRPR also helped develop the NIH Bicentennial exhibit in the new HEW Portal Building. NIAID's section of this exhibit is concerned with research on the hepatitis B vaccine.

The Institute co-sponsored a successful science writers briefing via long-distance telephone. Allergists attending the annual meeting of the American Academy of Allergists in San Diego, Calif., in San Diego were questioned by science writers gathered both in San Diego and on the NIH campus. A small exhibit at the Academy meeting displayed NIAID publications on immunology and allergic diseases.

At the National Academy of Sciences' Symposium on Viral Hepatitis, held in March, the ORRPR assisted in arranging a press briefing on the development of vaccines for hepatitis B. Two Institute scientists participated in this briefing which was well attended by members of the Washington science writing press.

This year, the Chief, ORRPR was designated to serve as the Institute's focus for Freedom of Information activities. An index of Institute documents has been developed under the guidance of the Institute's executive officer.

Maintained throughout the year has been an active program for dissemination of information on new knowledge and new biomedical materials that are the products of the research endeavors supported by the Institute. These program activities include classical methods for scientific communications and the development of new and improved techniques for rapid and frequent communications between biomedical research scientists.

The preparation and publication of formal research manuscripts, including page costs for printing and in some cases manuscript review costs, account for the major portion of the funding. Manuscripts and research notes submitted for publication during FY 75 were approximately 3000 from grantees, 150 from contractors and over 800 from NIAID staff. The total is nearly 20 percent higher than the previous year. In addition, a large number of abstracts, proceedings of scientific meetings, and progress reports from research contractors were prepared. Copies of the technical reports are made available through the Department of Commerce "National Technical Information Service".

Promotion of scientific communications existed also in the support of scientific meetings. Most of the 38 meetings funded by the Institute in FY 75 were workshops or small conferences wherein indepth discussion of a relatively narrow subject area occurred. These meetings were planned and conducted by the Institute as an integral part of program activity. In addition, the Institute received proposals or "meeting prospecti" from individuals in the scientific community seeking support for conferences, congresses and scientific workshops. Of 16 such proposals, a total of 10 meetings received, via contracts, at least partial support from NIAID, with 5 of those meetings jointly funded with other Institutes at NIH. By this arrangement, two, three, or four Institutes can provide funding for a meeting through a single contract document. In addition, 69 seminars were presented by visiting scientists to the intramural laboratories.

Satisfactory progress was gained in the program activities devoted to new and improved methods of scientific communications. The "Scientific Memoranda" for fast, frequent, and informal communications between research investigators were continued for the research areas of interferon, hepatitis, leprosy, hyper-sensitivity disease, transfer factor, and sexually transmitted diseases (formerly neisseria-treponema). Through this mechanism, over 1000 scientists located in 47 countries plus the United States were able to exchange research progress reports, abstracts and other pre-published information about current experiments. In June 1975, the "Nucleic Acid Recombinant Scientific Memoranda" was initiated for scientists studying recombinant molecules.

The experimental use of a NASA satellite for scientific communications continued throughout the year. The ATS-1 satellite located over the Pacific Ocean was employed for scientist to scientist communications and was especially useful to investigators located at isolated sites. Scheduled use of the satellite varied in days and hours from time to time and although the time difference between Bethesda, Md. and sites in the mid or western Pacific were considerable, effective communications could be maintained. During FY 75 a research team from NIAID spent a period of six weeks studying, in collaboration with the local authorities, filariasis on the island of Mauke in the Cook Islands. These studies received vital support through communications, via satellite, with laboratories in Hawaii and continental U.S.A. Plans were developed with the Lister Hill National Center for Biomedical Communications, NLM/NIH, for NIAID experiments on a new Canadian-American satellite (CTS) scheduled to be launched in December, 1975. This satellite will be focused on the North American continent and will have both video and audio capabilities.

As an outgrowth of the so-called Cooper Committee the OD/NIH established a number of subcommittees or task forces to work out the details of the conc problems and desirable procedures identified by the Cooper Committee. One o the subcommittees attempted to formulate a new NIH Manual Issuance 1810 -- Definition, Approval and Announcement of Programs. As part of the new program concept each Institute was requested to devise a Program Structure or outline of activities which, because of its branching nature, became known as the Program Tree.

Over a period of 3 months a continuing attempt was made to involve the NIAID professional staff in the extramural and collaborative programs as well as the Office of the Director. Instructions were simple:

1. The Program Structure should provide a framework on which both contract and grant activities can be displayed in some intellectually reasonable and coherent manner. Hopefully the framework will be such that L&CR activities can be incorporated at a later date.

2. The Program Structure is intended to serve as the conceptual basis for reporting on programs for such purposes:

    - in-house program review
    - Annual Report
    - RCAG background presentations in connection with specific proposals
    - budget narrative and other budget presentations
    - Special Reports to Congress
    - program presentations to Council and Building 1
    - program evaluation
    - categorizations of individual projects in programs
    - reporting to public (insofar as possible) on program effort
    - statements of level of research effort.

3. On one end the Program Structure is rooted in our two Program Budget Activities (Allergic & Immunologic Diseases, Infectious Diseases); on the other or operating end the structure should represent a reasonable concensus of the structure or categories which the operating personnel find useful in planning, reporting, presenting, aggregating and conceptualizing their program activities.

The constraints were few:

1. A branching tree structure with multiple levels or loci of branching was required by OD/NIH.
2. NIAID has two Program Activities -- Allergic & Immunologic Diseases and Infectious Diseases.
3. By definition (OD/NIH) L&CR was to be excluded from the Tree.

There was a series of formal and informal meetings with professional staff, the exchange of memos of comment, and several iterations of the "tree" with modifications to accommodate different perceptions. It cannot be said, however, that the tree concept or the program structure as sent forward to OD/NIH represented complete agreement and acceptance. In fact the memo of transmittal stated:

> "Although this version (1/27/75 Draft) is an evolutionary step in the method of displaying program and in our Institute's concepts of program it must still be regarded as tentative. The exercise of preparing the Tree provoked considerable thought and not a few reservations among the professional personnel responsible for operations at several levels. As an Institute we have not yet arrived at a genuine consensus as to the characteristics of a program, the interaction between various support and advisory mechanisms, nor the way in which these characteristics and interaction can best be displayed."

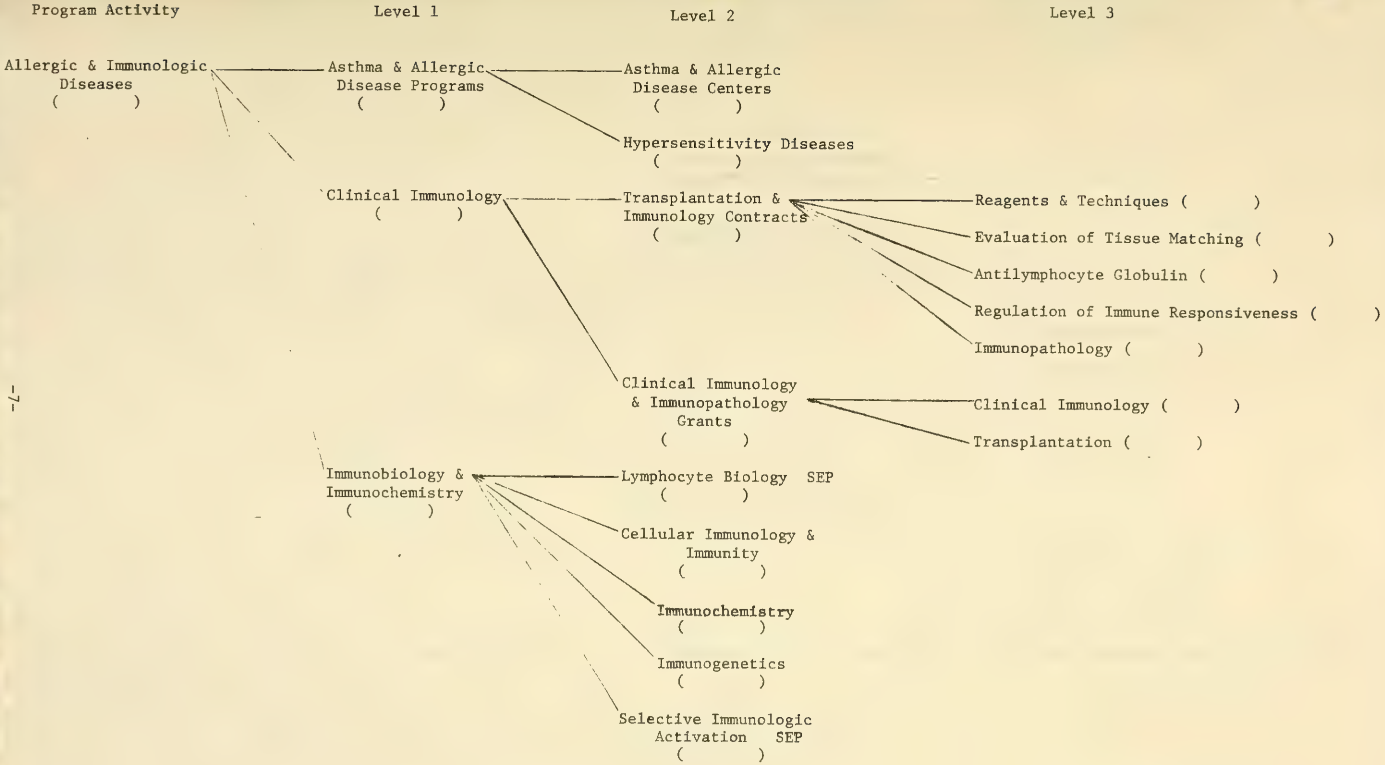
In anticipation that Manual Issuance 1810 would appear (it had not by June 30, 1975) and would require the appointment of program officials, there was some preliminary discussion of titles and functions at different levels. The following brief table summarizes these scanty and tentative suggestions.

PROGRAM LEVELS -- OFFICIALS AND FUNCTION

	Title	Function
Program Activity	Institute Director	Policy formulated Level 2 (? Level 1) Goals/Objectives approved Resources allocated
Level 1	Institute Program Coordinator	Coordinates Level 2 Programs
Level 2	Program Operations Manager or Officer	Operates or carries out policy

The first genuine test of the usefulness, acceptability and validity of the Program Tree came when it was time to write the Annual Report. The instructions to the reporting elements in the extramural and collaborative program were to organize the report by the program structure of the Tree. After a few discussions and modifications this did appear to be an easy outline to follow. And so the Program Tree (revision of 6/30/75) given below may be on the way to becoming a recognized, acceptable and useful device for displaying Institute-wide programs.

PROGRAM STRUCTURE OF NIAID[1]


Program Activity
Level 1
Level 2
Level 3
Allergic & Immunologic Diseases ( )
Asthma & Allergic Disease Programs ( )
Asthma & Allergic Disease Centers ( )
Hypersensitivity Diseases ( )
Clinical Immunology ( )
Transplantation & Immunology Contracts ( )
Reagents & Techniques ( )
Evaluation of Tissue Matching ( )
Antilymphocyte Globulin ( )
Regulation of Immune Responsiveness ( )
Immunopathology ( )
Clinical Immunology & Immunopathology Grants ( )
Clinical Immunology ( )
Transplantation ( )
Immunobiology & Immunochemistry ( )
Lymphocyte Biology SEP ( )
Cellular Immunology & Immunity ( )
Immunochemistry ( )
Immunogenetics ( )
Selective Immunologic Activation SEP ( )

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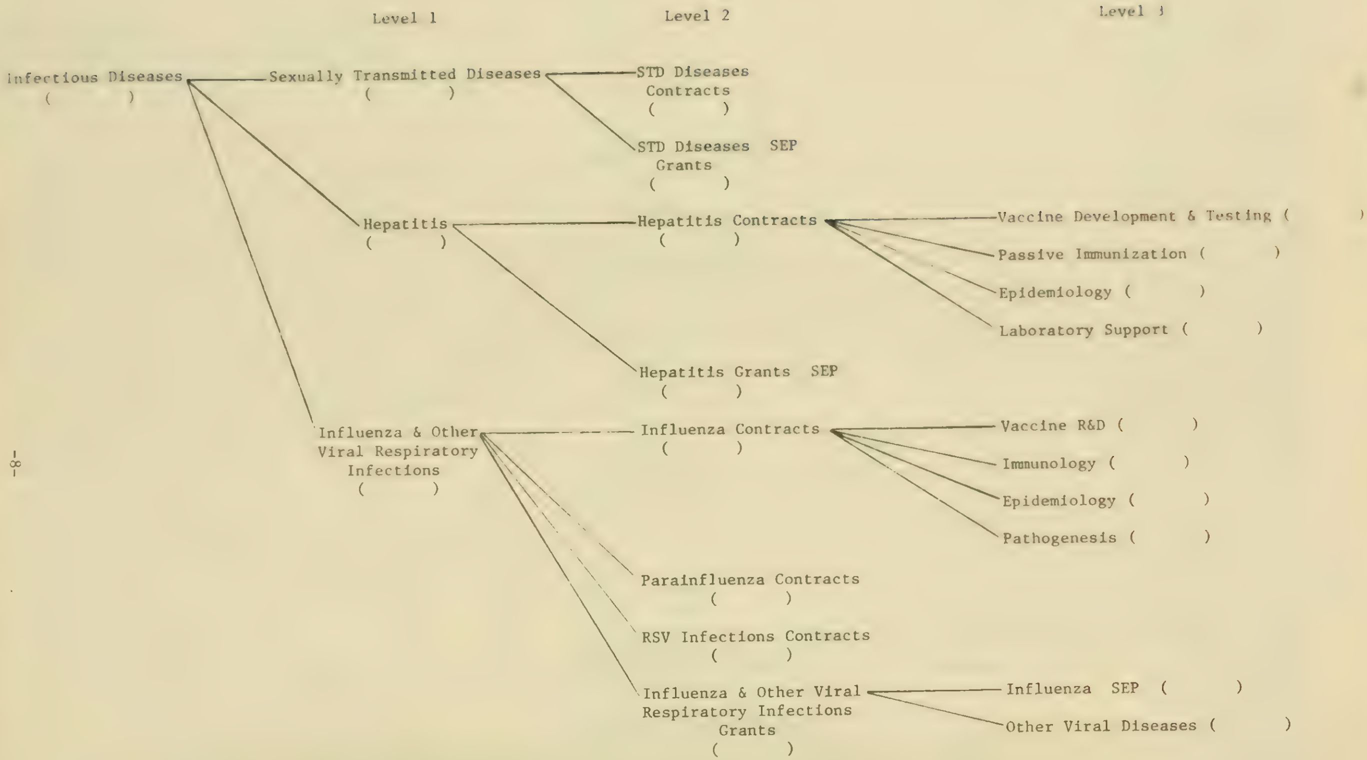
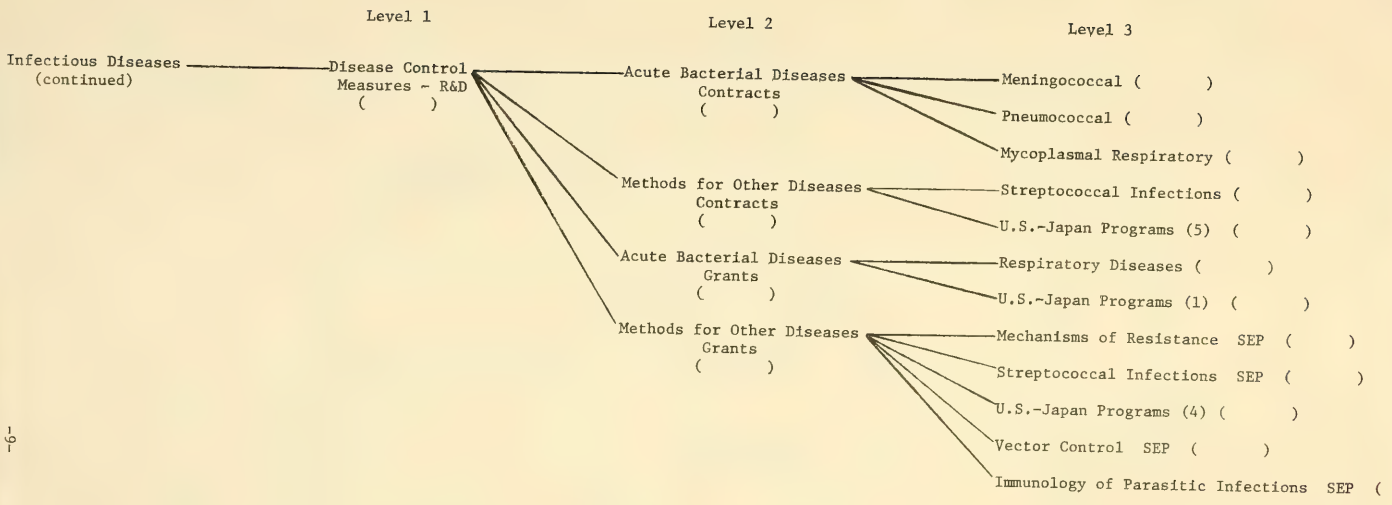
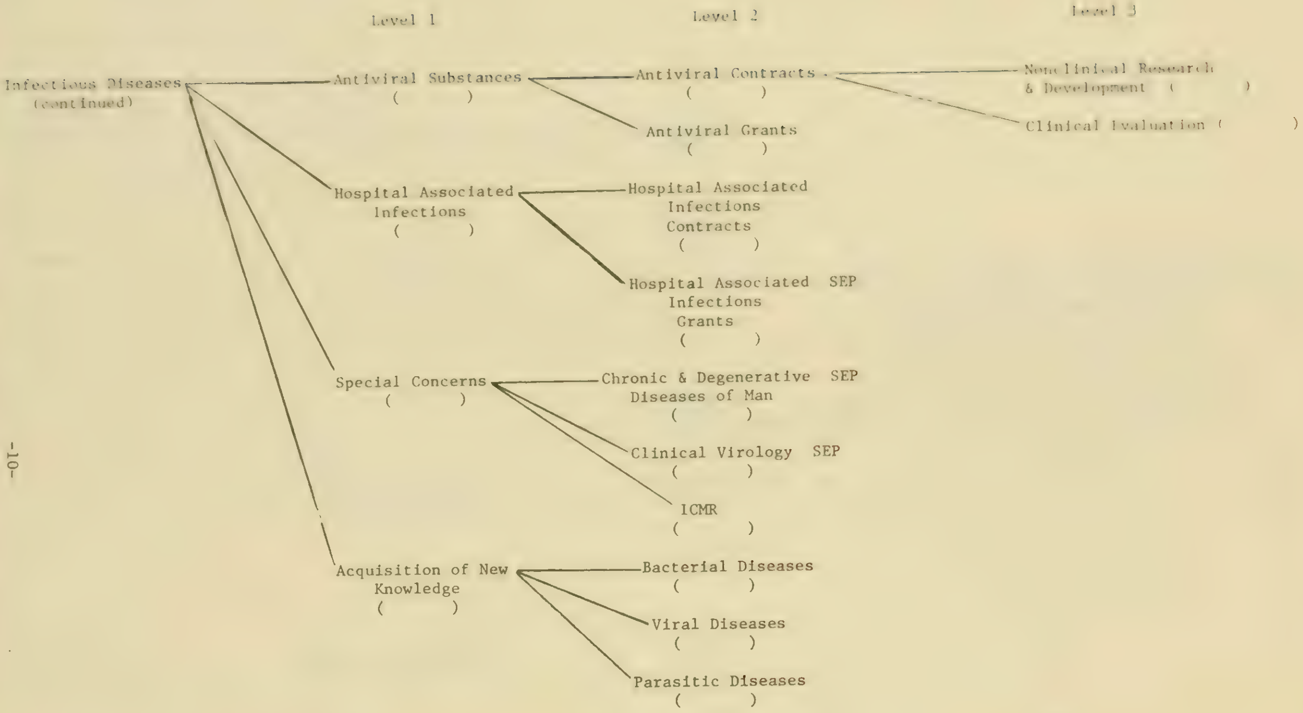
[1] FY75 obligations, dollars in thousands.
Grants includes grants and training awards.
Amounts cannot be added in the direction of Level 3 to Level 1.

June 30, 1975


Level 1
Level 2
Level 3
Infectious Diseases ( )
Sexually Transmitted Diseases ( )
STD Diseases Contracts ( )
STD Diseases SEP Grants ( )
Hepatitis ( )
Hepatitis Contracts ( )
Vaccine Development & Testing ( )
Passive Immunization ( )
Epidemiology ( )
Laboratory Support ( )
Hepatitis Grants SEP ( )
Influenza & Other Viral Respiratory Infections ( )
Influenza Contracts ( )
Vaccine R&D ( )
Immunology ( )
Epidemiology ( )
Pathogenesis ( )
Parainfluenza Contracts ( )
RSV Infections Contracts ( )
Influenza & Other Viral Respiratory Infections Grants ( )
Influenza SEP ( )
Other Viral Diseases ( )


Level 1
Level 2
Level 3
Infectious Diseases (continued)
Disease Control Measures - R&D (   )
Acute Bacterial Diseases Contracts (   )
Meningococcal (   )
Pneumococcal (   )
Mycoplasmal Respiratory (   )
Methods for Other Diseases Contracts (   )
Streptococcal Infections (   )
U.S.-Japan Programs (5) (   )
Acute Bacterial Diseases Grants (   )
Respiratory Diseases (   )
U.S.-Japan Programs (1) (   )
Methods for Other Diseases Grants (   )
Mechanisms of Resistance SEP (   )
Streptococcal Infections SEP (   )
U.S.-Japan Programs (4) (   )
Vector Control SEP (   )
Immunology of Parasitic Infections SEP (


Level 1
Level 2
Level 3
Infectious Diseases (continued)
Antiviral Substances ( )
Antiviral Contracts ( )
Nonclinical Research & Development ( )
Clinical Evaluation ( )
Antiviral Grants ( )
Hospital Associated Infections ( )
Hospital Associated Infections Contracts ( )
Hospital Associated Infections Grants ( ) SEP
Special Concerns ( )
Chronic & Degenerative Diseases of Man ( ) SEP
Clinical Virology ( ) SEP
ICMR ( )
Acquisition of New Knowledge ( )
Bacterial Diseases ( )
Viral Diseases ( )
Parasitic Diseases ( )

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EXTRAMURAL PROGRAMS - NIAID

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(SEP - Special Emphasis Program)

REPORT OF THE ASSOCIATE DIRECTOR

Introduction

Fiscal Year 1975 was the year of the three R's in extramural programs: rescission, regulations and reports. As in recent fiscal years, the appropriation process was able to continue its tradition of producing an unusual year. In this fiscal year, the President's budget and the Congressional appropriation varied by some $14 million. This controversy involved an effort by the Executive Branch to institute a rescission of the appropriation and a counter effort by the Congress to deny this rescission. By the beginning of the last quarter of the fiscal year, the Congress prevailed and we were able to continue the funding of applications through approximately the 45th percentile, as we did in FY 1974, following the release of impounded funds.

The most labor-intensive new activities of FY 1975 were the new regulations and the special reports requested. Seldom were the two related. There were new regulations and procedures instituted for consideration of research projects involving human subjects or biohazards. Committee Management and the appointment of consultants became more complex. Increased accessibility to investigator-developed information and more complete documentation of advisory committee meetings are required by the Freedom of Information Act.

Fiscal management and budget planning required tracking of funds by both program and by award instrument. There were over 40 special reports requested by departmental sources--a considerable increase over FY 1974.

A further complication for fiscal planning in fiscal year 1975 concerned training. It was May 5 before the issue of whether we would support institutional fellowships and whether we might support predoctoral training was decided. The NIAID had not requested authority to support predoctoral training during FY 1975; nevertheless delay of the decision on all training support added to the problem of accomplishing all training awards in the last eight weeks of the fiscal year.

Human Subjects

DHEW policies and procedures on the protection of human subjects were published in the Federal Register on May 30, 1974. These did not differ substantially from the original policies but did emphasize the need for the applicant institutional committee to review any activity involving human subjects and provide the National Institutes of Health with a certification of such review and approval, before such activity can be funded by the DHEW. A memorandum was released from the Office of the Associate Director for Extramural Research and Training in June 1974 specifying that, after July 1, 1975, organizational review and approval must be completed and certified prior to submission of proposals to DHEW. During the last fiscal year, institutions were allowed a 30-day grace period from the time of submission of the proposal within which a certification might be submitted. On

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August 1, 1974, a flyer was inserted in all application kits entitled, "Research Projects Involving Human Subjects," which required that in proposals involving human subjects the applicant, under Methods of Procedure:

1. Describe the requirements for a subject population, explaining the rationale for using in this population special groups such as children and prisoners.

2. Describe and assess any potential risks.

3. Describe consent procedures to be followed.

4. Describe procedures for protecting against or minimizing potential risks.

5. Assess the potential benefits to be gained by the subject.

6. Analyze the risk-benefit ratio.

P.L. 93-348, signed into law on July 12, 1974, established a National Commission for the Protection of Human Subjects to study the problem of human experimentation and to submit recommendations to the Secretary, DHEW, within two years. This report would form a basis for future DHEW policies. A moratorium was also issued banning all research on living fetuses until such time as the National Commission makes recommendations to the Secretary. On April 27, 1975, the Commission recommended a lifting of the moratorium and to allow research to proceed under current regulations of the DHEW.

A new form (HEW 596-rev.'2/75) has replaced the old human certification form. The new one is more detailed and includes among other things, a declaration as to the use of special subject groups, an investigational new drug certification (if applicable) and a special section for studies involving cooperating institutions.

An announcement was made on April 14, 1975, that the Division of Research Grants has developed a computerized tracking system for identifying projects involving human subjects.

All these new policies will help to settle questions and expedite the orderly processing of applications involving human subjects. They will also stress to the applicant institutions the seriousness of NIH's intent to make sure that the DHEW regulations on human subjects are being properly implemented.

NIAID has had a long-standing policy of identifying for Council discussion each research grant application wherein human risks, real or potential, are identified by the study sections. These concerns are communicated to the investigator and the grantee Institutional Review Committee. Many of the questions have stemmed from inadequacy of information in the application.

Hopefully, these new policies will diminish the number of such problem applications.

In a continuing effort by DHEW to identify federal research support which has relevance to clinical care, all Institutes were asked to make an inventory of NIH-supported clinical trials. As of March 1975, EP-NIAID was supporting 173 clinical research projects, amounting to about $11.6 million, which constitutes 17.2% of the total EP investment in research projects. For all of NIH, the amounts are $160.9 million for clinical trials and $645.7 million, for clinical research projects. Thus in FY 1975, the NIH investment of $645.7 million on clinical research is roughly a third of the total NIH budget.

Again, as part of a DHEW effort, the NIAID has been requested this year to identify extramural research efforts pertaining to the health of mothers and children, and a very recent survey that is in process requests EP-NIAID efforts relevant to infants of low birth weight.

Freedom of Information

The 1974 Freedom of Information Act, as presently interpreted, permits access of information to any person requesting it on all approved and funded research grant applications. Exempt from disclosure are unapproved, approved but unfunded, applications, study section summary statements, and site visit reports. The Privacy Act of 1974 restricts disclosure of information on an individual without the written consent of that individual. It overlaps with the Freedom of Information Act to some degree, but will not become effective until late this year. Records must be kept on all information requested.

NIAID-EP has received two requests for material from the same requestor. On each occasion, we have provided the material within the 24 hours after the receipt of the memorandum from the Office of the Director, NIH. To date, no denials have been finalized, however two grantees requested that their proposals not be made available because of possible patentable material. These matters were forwarded to the NIH Patent Advisor's office.

Program Evaluation

The program evaluation activity of Extramural Programs continued in fiscal year 1975 at approximately the same level as in 1974. Three programs were evaluated. These programs were: Immunity to Parasitic Infections; the Biological Regulation of Vectors; and the Mechanisms of Resistance to Antimicrobial Agents. The program on Biological Regulation of Vectors was evaluated at the end of FY 1974, and was reported to the June 1974 Council. A detailed discussion of these evaluations will be found under the report on each program.

A complete discussion of the program evaluation activity was presented to the November NIAID Council by Dr. Wilford Bailey, who had participated most actively with Dr. Gay in the evaluation program, and had served as chairman of the evaluation group for Immunity to Parasitic Infections and Biological Regulation of Vectors.

Five of the Special Emphasis Programs have been evaluated to date. These programs have averaged approximately 30 projects each. In the more recent evaluations, the principal investigators were asked to provide a special progress report. They were more than willing to do this and have modified the organization of their final progress report to take cognizance of the goals and objectives of the Special Emphasis Program. These goals and objectives were furnished the principal investigators at the time we asked for the special progress reports. Information for the evaluation review has consisted principally of staff-furnished data on program support, the Council-approved program descriptions, goals and objectives, and the above-mentioned special progress report.

The practice of providing a report of the evaluations of Special Emphasis Programs to each Principal Investigator whose project was assigned to the program was continued. We have not yet evaluated the effect of this action on the renewal applications of program grantees. In the near future, there should be enough of these applications received for review that it will be possible to study the report's impact.

The evaluation appears to have been useful in the planning and programming of the Special Emphasis Programs and in the soliciting of new applications. It has been another way of highlighting to the scientific community the special emphasis the Institute wishes to place on selected facets of its research program.

The evaluations have prompted small organizational changes and only minor modifications in the goals and objectives of the Special Emphasis Programs. The evaluators have concerned themselves primarily with the scientific content, organization, and progress of the program.

More clear-cut program assignment of applications has resulted from the program evaluation, although there have been only few assignment disagreements between evaluation consultants and staff to date.

In addition to minor modifications about goals, objectives, an [illegible] program content, the evaluators have suggested needs for national meetinc [illegible] two of the program areas to review problems such as program developme [illegible]logical hazards. They have also identified many program accom [illegible] ments which may be suitable for further research or development.

During the coming year, it would be appropriate to continue the [illegible] ion program, examining the Venereal Disease program, the Asthma and [illegible]ergic Disease Centers, and the Viral Hepatitis program. It would also be appropriate to have a management review of the evaluation process as it is now practiced. This review would consider needed changes in the process before our other Special Emphasis Programs are evaluated, and before we try to expand this evaluation process to cover larger, less discrete programs.

Training and Fellowships

Since the last annual report was submitted, the training programs administered by the NIAID have changed in terms of authority and character.

It is appropriate to briefly review the old programs; that is, those programs now in the process of being phased out. Under section 301(d) of the Public Health Service Act, Title III, Public Law 78-410 (1970), the objectives were to assist public and nonprofit institutions in establishing, expanding and improving training opportunities for individuals interested in research training in areas of scientific concern to the NIAID. That program, an institutional program, was identified by program code "T01." Support was afforded to the successful institutions to assist both predoctoral and postdoctoral trainees.

During early 1974, there was a release of impounded funds which increased the funds available for support to these institutions. The net effect of this release of funds on training will not be known for some time, inasmuch as progress reports from the various institutions will not be available until later this calendar year.

Currently, there are 73 "T01" programs which are still active. However, 23 of these will terminate as of June 30, 1975. The remaining 50 "T01" programs have varying periods of time to be phased out, the last terminating in FY 1978. About 500 trainees were supported in each of the last three years by this mechanism. Under this same authority, P.L. 78-410, the NIAID also supported other fellowship programs designated by program codes "F02" and "F03." These programs are also in the process of being phased out.

In the summer of 1973, instructions were issued by the Secretary's office to initiate new postdoctoral programs to replace the "T01," "F02," and "F03" programs. These replacements were program coded "T22" for the Institutional postdoctoral training only. Whereas all Institutes participated in the Individual Fellowship program, only four Institutes--of which NIAID was one--participated in the Institutional Fellowship program. The NIAID currently supports eight "T22" Institutional Fellowship programs which have a total of 16 trainees and supports 90 Individual postdoctoral fellows. This program will also be phased out within the next two years.

The "F22" and "T22" programs were superseded by the new National Research Service Award Act of 1974 which became law on July 12, 1974, as Public Law 93-348. The purpose of this law is to consolidate all research training authorities of the NIH and the Alcohol, Drug Abuse, and Mental Health Administration (ADAMHA). In furtherance of this purpose, the Congress also repealed existing research training and fellowship authorities of the ADAMHA and components of NIH through a series of conforming amendments.

The November 1974 Council reviewed a total of 256 "F32" applications. Of these, 248 were approved, seven were disapproved, and one was deferred.

The latest receipt date of February 15 for Individual Fellowship and Institutional Fellowship applications resulted in the receipt of a total of 180 Individual and five Institutional Fellowship applications which have been reviewed.

The NIAID will be supporting Fellowship and Training for an estimated $8.96 million in FY 1975. The noncompeting component of this amount, including the phase-out training programs "T01" and "T22" and the fellowships "F02," "F03" and "F22," will be approximately $6.2 million, whereas the competing component, the "T32" and F32" programs will be approximately $2.7 million. This means that for this fiscal year approximately 154 new fellows and eight new Institutional Fellowship programs will be funded.

Committee Management

Committees and the operation and use of advisory groups have been under close scrutiny since the passage of the Federal Advisory Committee Act in the fall of 1972, and even more stringent regulations are now being enforced. Advisory groups giving consensus advice to any segment of the federal government must be chartered as a committee, and notices of their meetings must be published in the Federal Register well in advance of the meeting. All meetings are open to the public except in those instances where a determination is made to close the meeting for the specific purpose of reviewing or discussing matters or documents of a proprietary nature, citing pertinent sections of Title 5, U.S. Code.

To insure compliance with the Federal Advisory Committee Act concerning conflict of interest, regulations have been issued this year for the conduct of NIH committees. To prevent even the appearance of a conflict of interest, committee members may not be present while the committee is discussing or voting on an application from any component of a state system with which the committee member has any type of affiliation. Just prior to adjournment of the meeting, all committee members are now required to sign a certification that they in fact did not discuss or vote on such applications.

The National Advisory Allergy and Infectious Diseases Council has been operating for several years with a number of vacancies. During FY 1975, seven new members were appointed by the Department; one appointee declined the invitation. At present there are only two vacancies on the Council out of the authorized 15 appointed members.

The authorized membership of the Allergy and Immunology Research Committee was increased from 10 to 12 in 1974. An appointment has been made for one of these vacancies, and appointments have already been made for four vacancies which will be created on June 30, 1975 by retiring members.

Biohazards

The Initial Review Groups of NIH have been requested to identify those research grant proposals in which a biohazard from potentially infectious agents exists or could become a potential problem. The biohazard problem is distinct from that of patients at risk as part of a research project. Biohazards identify the dangers--actual or potential--of laboratory procedures to the investigator, his staff, or to casual bystanders. The NIH will communicate these concerns regarding biohazards to the investigator and the grantee institution; a firmer policing action is not envisaged at this time. The NIAID will participate fully in development of policies and procedures of the NIH regarding the biohazard problem; it will play an active role in future conferences or planning sessions in which operating procedures or guidelines may be adopted.

During this fiscal year, a number of scientists have become concerned with possible biological hazards from genetic manipulation or recombinant DNA arrangements. The Department has chartered a special advisory committee to look at this problem. A further discussion of this potential problem is found in the discussion of Recombinant DNA and Genetic Engineering, page 70.

Personnel

During the last year, we have had to continue to operate with limited personnel resources, but through the adjustment of some responsibilities to utilize existing professional staff and through new appointments in professional positions, we have been able to develop a staff with the expertise and abilities necessary to carry out our responsibilities. New staff appointments have been made with an eye toward our future needs in the areas of basic immunology, virology and manpower development programs.

In July, Dr. Wilford Bailey, Chief, Parasitology and Medical Entomology Branch, returned to his former position in the School of Veterinary Medicine at Auburn University. The responsibility for the parasitology and medical entomology programs was assumed by Dr. Irving Delappe in addition to his continuing responsibilities as Chief of the Biochemistry and Physiology Branch.

Dr. Noel Gross, who had served as Special Assistant for Training Programs for the last 15 years, retired after twenty-six years of distinguished Federal service. Dr. Gross had also served the NIAID Extramural Programs as Chief of the Bacteriology and Mycology Branch from 1965 through 1969.

Dr. Louis Bourgeois joined our staff in October 1974 as Assistant for Research Manpower Development Programs. He had previously served as Program Consultant in the Division of Associated Health Professions, Bureau of Health Resources Development.

Dr. Bernard Janicki, former research investigator and Chief of the Pulmonary Immunology Research Laboratory at the Veterans Administration Hospital in Washington, D.C., has joined our staff to assist Dr. Sheldon Cohen in the

Allergy and Immunology Branch. Dr. Janicki's primary area of responsibility is in the basic immunology programs.

We have also been fortunate in being able to appoint Dr. William Allen to assist Dr. Clarence Sooter in developing and administering the viral diseases programs. Dr. Allen had previously engaged in viral research and served as Head of the Pathobiology Division and Virology Department at the Delta Regional Primate Research Center, Tulane University. He had also served as Chief, Virology IV Branch, Virus and Rickettsia Division, U.S. Army Biological Laboratories, Fort Detrick from 1967 to 1971.

Approximately forty-five percent of our staff participated in formal training programs. Twenty-two employees attended a total of forty-two courses at a cost of $2400. The cost does not include the salaries while training, nor support of the STEP courses or the Upward Mobility Program which are funded centrally.

Cash awards were made to the staff of the Grants Management and Operations Branch and to those members of the Review and Evaluation Branch engaged in the budget and fiscal reporting functions. They were cited for their efficiency and dedication in handling the unusually heavy workload at the end of FY 1974. This type of performance on the part of our support staff has enabled us to operate efficiently in spite of changing requirements, extreme fluctuations in workload and an increase in applications processed during FY 1975. (See Figure I)

Program Analysis

There has been an increasing demand on the Program Analysis Section, Review and Evaluation Branch, to provide programmatic data and analyses, not only to the NIAID for the purpose of program management and evaluation, but also to other B/I/D's at the NIH, the DHEW, other government agencies, and members of Congress. During the past year, there have been over forty special requests for program information, involving both the research and training areas; more than half of these requests were initiated outside the NIAID by other parts of DHEW. This represents a substantial increase in special requests, particularly regarding those requests initiated from outside the Institute. Some reports involved extensive analyses of our grant data, for example: an inventory of NIH supported clinical trials requested by DRG for the Assistant Secretary of Health, involving over three person-months of professional effort since the fall of 1974. Our contribution to a report on DHEW research effort pertaining to the health of mothers and children requested by NICHD for DHEW represented another substantial investment of effort in the program analysis area.

Fiscal Management and Reporting

In FY 1975, there was a dramatic shift in emphasis in the Extramural Programs from management and control of funds by mechanisms (research, training, and fellowships) to management and control of funds by the four major disease programs of the Institute. One of the first steps undertaken to make budget data retrievable by program areas was to assign codes to all active grants and awards plus all pending applications approved by Council for funding. At the same time, the accounting and bookkeeping system of the Institute was completely restructured to allow for the development of the budget along program lines in addition to reflecting the type, number and dollars within each of the various mechanisms. This system was also designed to permit rapid reporting and accurate accounting of grants and awards in each of the subprograms including, among others, the special emphasis areas. The interrelationship between program, subprogram and mechanism codes is illustrated in TABLE II.

In developing the coding and accounting systems, an allowance was provided for making program changes as the need arises. The first major change will be made in FY 1976 when three of the four major disease programs (Bacterial and Fungal Diseases, Viral Diseases, and Parasitic Diseases) will be incorporated into one program, Infectious Diseases. The flexibility which was built into the system at the onset should allow this change with a minimum of procedural problems.

TABLE I

EP-NIAID COMPETING GRANTS AND AWARDS

REVIEWED/APPROVED/FUNDED

	FY 74 Incldg. FY 73/74	FY 75 (Est'd)
RESEARCH GRANTS:		
Reviewed	976	1,031
Approved	787	847
Funded	425 1/	375
Percent of Approvals Funded	47% 2/	44%
CAREER/ACADEMIC AWARDS:		
Reviewed	23	100
Approved	16	69
Funded	26 1/	22
FELLOWSHIPS (INDIVIDUAL):		
Reviewed	322	430
Approved	266	400
Funded	90	154
FELLOWSHIPS (INSTITUTIONAL):		
Reviewed	25	13
Approved	18	9
Funded	8	6

1/ Includes reactivated applications which requested FY 73 funds originally

2/ Represents percent of FY 74 approvals funded

TABLE II

NIAID Extramural Management Code Categories

A. Major Disease Headings and Subdivisions:

8425600 Allergic and Immunologic Diseases
- 60_ Asthma & Allergic Disease Centers
- 61_ Clinical Immunology
- 62_ Selective Activation of Cell Mediated vs Humoral Antibody Responses
- 63_ Lymphocyte Biology
- 68_ Other (M-1 Program)

8425700 Bacterial and Fungal Diseases
- 70_ Venereal Diseases
- 71_ Bac. Dis. Preventable by Vaccines
- 72_ Hospital Associated Infections
- 73_ Mechanisms of Resistance to Antimicrobial Agents
- 78_ Other (M-1 Program)

8425800 Viral Diseases
- 80_ Influenza & other Respiratory Dis.
- 81_ Hepatitis
- 82_ Antiviral Substances
- 83_ Clinical Virology
- 84_ Chronic & Degenerative Diseases
- 88_ Other (M-1 Program)

8425900 Parasitic Diseases
- 90_ Immunity to Parasites
- 91_ Biological Regulation of Vectors
- 98_ Other (M-1 Program)

B. Extramural Mechanisms:
- _1 Regular Research Grants
- _2 Categorical Clinical Centers
- _3 Research Career Program
- _4 Research Training Program (Individual)
- _5 Research Training Program (Institutional)
- _6 Fellowships (Old)
- _7 Training Grants

EXAMPLE: A regular research grant in Venereal Diseases would be coded as follows:

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PROGRAM
┌─────────
│   SUBPROGRAM
│   ┌──────────
│   │   MECHANISM
│   │   ┌─────────
7   0   1
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TABLE III

PROGRAM ANALYSIS

FY 1975 - REGULAR RESEARCH GRANTS
(Dollars in thousands)

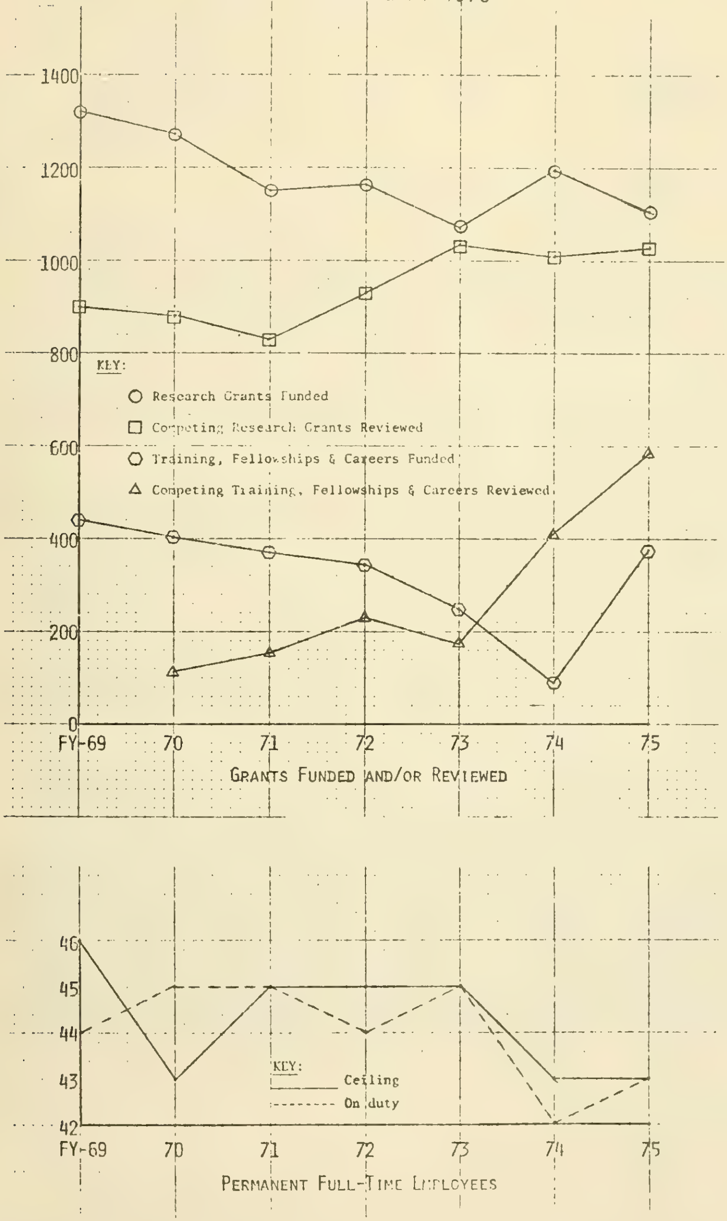
PROGRAMS	TOTAL AMOUNT 1/
ALLERGIC AND IMMUNOLOGIC DISEASES:	$ 25,300
Asthma and Allergic Diseases	1,898
(Asthma and Allergic Disease Centers ....$ 1,429)	
Clinical Immunology and Immunopathology	4,705
Selective Immunologic Activation	2,157
Lymphocyte Biology	1,629
BACTERIAL AND FUNGAL DISEASES:	18,900 2/
Biology of Venereal Diseases	2,069
Bacterial Diseases Preventable by Vaccines	3,267
Hospital-Associated Infections	1,643
Mechanisms of Resistance to Antimicrobial Agents	1,938
VIRAL DISEASES:	13,700
Influenza and other Respiratory Diseases	1,218
Viral Hepatitis	324
Antiviral Substances	1,011
Clinical Virology	513
Chronic and Degenerative Diseases of Man	1,130
PARASITIC DISEASES:	6,600
Immunology of Parasitic Infections	1,744
Biological Regulation of Vectors	1,956
TOTAL REGULAR RESEARCH GRANTS	$ 64,500

1/ Estimated

2/ Includes Grants Administered by the Biochemistry and Physiology Branch.

Figure I

NIAID Grants Funded and/or Reviewed -- Manpower
FY-1969 thru FY-1975



I. ALLERGY AND IMMUNOLOGY BRANCH

The approximate level of support in allergy and immunology research:

Type of Grant	Number	Amount
Research Grants 1/	410	$25,829,817
Training Grants	36	1,805,906
Fellowships	74	856,475

A. Asthma & Allergic Diseases Centers (SEP)

There are currently 16 Asthma and Allergic Disease Centers supported by this NIAID program at a level of funding totaling $1,428,558 with a geographic distribution covering 13 states. Each represents a collaborative endeavor among basic and clinical medical scientists concerned with investigations on the etiology, diagnosis and treatment of asthma and allergic diseases. Laboratory research findings in immunologgy, biochemistry and pharmacology are being translated into clinical studies with relevance and applicability to hypersensitivity diseases of man in institutions where especial expertise and effective facilities can be identified.

The Center at Robert B. Brigham Hospital and Harvard Medical School, Austen, K.F., AI 10-356-04, has been concerned with mechanisms of immunologic release of chemical mediators of immediate and delayed hypersensitivity and the inter-relationships of clotting factors and inflammation. This group has been able to: (1) define the action of complement pathways in patients with cutaneous vasculitis, angioedema and defects in host resistance effected by leucocyte mobilization factors, and in immunotherapy, (2) chemically characterize slow reactive substance of anaphylaxis, and (3) delineate pathophysiologic mechanisms responsible for respiratory tract diseases with eosinophilic infiltrations.

The SUNY at Buffalo Center, Arbesman, C.E., AI 10, 397-03, concerned with insect sting reactions has: (1) isolated five purified fractions from bee, yellow jacket and hornet venoms, (2) developed RAST as an efficacious diagnostic method, (3) identified phospholipase A as the primary allergen venom, and (4) established the efficacy of immunotherapy with bee venom over that of the use of whole body extract. Working in the same area the Johns Hopkins Center, Norman, P.S., AI 10,304-04, demonstrated lack of antigenic cross-reactivity of honey bee phospholipase A with venoms of other vespids and the inability of injections of whole body extracts to effect production of blocking antibody characteristic of naturally induced immunity to venom stings.

1/ Includes 54 career awards ($1,371,301)

Several Centers have been studying bronchospastic responses in asthmatics from both etiolgic and pathophysiologic standpoints. Findings of the Johns Hopkins group, Norman, P.S., AI 10,304-04, indicate that parasympathetic reflexes do not mediate allergic bronchospasm in man, and that with lung and skin mast cells and circulating basophils responding as a single population of mast cells to ragweed quantitative skin tests and inhalational bronchial challenge are equally useful for diagnostic purposes. In defining subsets of asthmatic types the Scripps Clinic and Research Foundation Center, Vaughan, J., AI 10,386-05 was able to correlate rises in plasma histamine levels and activation of complement pathways with both natural history and induced bronchospasm. The National Jewish Hospital Center, Farr, R.M., AI 10,398-04, has established parameters for utilizing bronchial challenge responses as a means of distinquishing clinically significant skin tests in the asthmatic. They have also identified relative ozygen lack as the responsible factor for ventilatory drive in response to bronchospasm.

The Center at Creighton University, Townley, R.G., AI 12,029-02, has developed inhalational challenge response tests to histamine and methacholine as bronchial sensitivity markers to predict the natural course of allergic airway disease and susceptibility and high risk for the development of asthma. The University of Michigan Center, Mathews, K.P., AI 10,391-04 in studying upper airway challenge to allergens has demonstrated that pharmacologic effects on nasal airway resistance are opposite to those on bronchi as a function of the vascular response in affected mucosal membranes.

Studies on pharmacologic modulation of hypersensitivity reactions have offered insight into mechanisms of human allergic disorders. The Washington University Center, Parker, C.W., AI 10,405, has identified alterations in cyclic AMP responsiveness in patients with chronic skin diseases, e.g. atopic dermatitis (allergic eczema) and psoriasis and prostaglandin (PGE_1) function in blood cells of asthmatics. Related studies at the University of Wisconsin Center, Reed, C.E., AI 10,404-04, identified the existence of high levels of cAMP in leucocytes and epidermal cells of atopic dermatitis. The work of the Children's Asthma Research Institute and Hospital Center, Middleton, E., AI 10,400-04, has demonstrated a spectrum of abnormalities in metabolism of cAMP and cGMP reflected in the leucocytes of asthmatic patients. In turn these findings offer leads for the rational use and development of new drugs to correct underlying biochemical lesions. The Johns Hopkins Center, Norman, P.S., AI 10,304-04, has also demonstrated deficiencies of lymphocyte cAMP rises in asthmatic patients.

Three Centers have been concerned with specific immunotherapy in allergic disease utilizing newly developed agents in ragweed hay fever as a prototype disorder. The Johns Hopkins Center, Norman, P.S., AI 10,304-04, demonstrated the superiority of results and enhanced blocking antibody production with treatment utilizing an allergoid preparation over that of conventional pollen extract. The work of the Northwestern University Center, Patterson, R., AI 11,403-01, has found that better results with reduced time and treatment required can be effected with a polymerized purified antigen E preparation. This group and the Mayo Clinic Center, Gleich, G.J., AI 11,483-01, have also developed effective in vitro assay systems for antibody-specific IgE enhancing the diagnostic accuracy of allergic diseases.

Allergists have always considered the diseases with which they deal to be based upon humoral immunologic mechanisms of immediate hypersensitivity. It is therefore of unusual interest to note research that is looking into the role of previously unsuspected cell mediated immunity (CMI). The work of the Duke University Center, Buckley, R.E., AI 12,026-02, supports the possibility that excessive IgE antibody production may be associated with deficient thymus-derived immune function in atopic patients manifesting eczema and that such depressed CMI may bear on their susceptibility to infection with viral agents. The University of California Center, San Francisco, Frick, O.L., AI 11,010-01, has contributed data to favor the presence of a delayed hypersensitivity (CMI) leucocyte inhibition factor in children with specific food sensitivities.

The relationship of infection to allergic diseases continues to be explored. The group at the University of Wisconsin Center, Reed, C.E., AI 10,404-04, has found that upper respiratory infections especially influence the small bronchial airways. By correlating this with viral induced effects on leucocyte cAMP responses effective and rational treatment for the identified biochemical abnormality can be determined. In the case of children exhibiting undue susceptibility to infection associated with hyperimmunoglobulinemia E the Duke University group, Buckley, R.E., AI 12,606-02, has identified CMI defects in lymphocyte and macrophage function of these patients. Clinical investigation at the University of Colorado Center, Claman, H.N., AI 12,028-02, support the suspicion that systemic vasculitis is an immune complex disease and that streptococcal infections may play a defined and pertinent etiologic role.

New developments in drug treatment for asthma have been advanced. These include work at the University of Wisconsin Center, Reed, C.E., AI 10,404-04, showing inhibitory relationships of the standard drugs ephedrine and theophylline and the therapeutic efficacy of new agents, an isopropyl derivative of atropine and a triamcinolone aerosol for bronchodilatation. Important contributions from the National Jewish Hospital at Denver Center, Farr, R.S., AI 10,938-04, include data on the efficacious anti-asthmatic steroid sparing effect of an antibiotic, troleandomycin (TAO), and an alpha adrenergic blocking agent, phentolamine, in exercise-induced bronchospasm.

The work of the Center at Louisiana State, Salvaggio, J., AI 10,975-02, has been directed to hypersensitivity pneumonitis especially related to exposure to fungal spores and industrial chemical air pollutants, e.g. toluene diisocyanate (TDI), sugar cane bagassosis and pulmonary silicosis. A high incidence of immune responses to airborne fungi has also been indentified, assays developed to implicate their significance in allergic lung diseases, and enzymatic and histamine-release effects demonstrated to suggest possible pathogenic mechanisms involved and in turn control measures.

1. Hypersensitivity Diseases

A programmatic area of especial interest and relevance to the mission of NIAID is that dealing with hypersensitivity diseases. Within this category are projects involving basic research, development and studies of animal models of human sensitization phenomena and clinical investigations of the allergic diseases. Subjects of ongoing study relevant to the function of this program are atopic mechanisms, allergic disorders, immediate hypersensitivity, allergens and allergenic inductive factors, IgE functions and disorders, anaphylactic sensitization and reactions, chemical and pharmacologic mediators of sensitization and inflammation, cell mediated delayed hypersensitivity reactions and specific immunotherapy of defined allergic diseases. Apart from the Asthma and Allergic Disease Centers Program, support of investigations in hypersensitivity diseases through project grants totals $2,563,000.

AI-08270-08 and AI 11,334-03, Lichtenstein, L.M., Johns Hopkins University. (1) Rather than finding deficient IgA in nasal secretions of hay fever patients, both IgA and IgG antibodies to ragweed antigen E were demonstrated. Increases followed immunotherapy; IgG blocking antibody being produced locally. (2) The presence of histamine has been shown to inhibit histamine release by increasing cAMP cellular levels acting at H-2 receptor sites. (3) Histamine release utilizing peripheral blood basophils has been feasibly adapted for use as a discriminating test to differentiate patients allergic to bee venoms. (4) The identification of phospholipase A as the major allergen in bee venom with the demonstration of antigenicity of mellitin and hyaluronidase in some instances allowed for the development of RAST assays for corresponding IgG antibody production in in treated patients.

AI 11,3[illegible]6-01, Adkinson, N.F., Johns Hopkins. A RAST test for the detection of anti-penicillin antibodies has been developed offering possibilities for valid prediction of penicillin allergy.

AI-09565-06, Marsh, D.G., Johns Hopkins. (1) Through collaborative endeavor the chemical nature of allergenic and antigenic determinants of ragweed allergen Ra5 has been ascertained. (2) A ragweed pollen derivative with reduced allergenic reactivity and retention of native antigenic properties (allergoid) has been successfully prepared and in clinical trials with P.S. Norman, AI 04866-13, show a trend for these treated patients to be improved over results obtainable with the use of conventional extracts. (3) Family studies on the ability to synthesize IgE indicated that this genetic ability can profoundly influence the development of specific allergies and established the interrelation between IgE levels and HL-A associated Ir genes for a complex allergen such as Group I rye grass pollen.

AI-01303-09, Arbesman, C.E., SUNY at Buffalo. (1) Evidence for an association between allergy and serous otitis media was suggested by the finding of IgE in affected patients' otic secretions. (2) Elevated serum IgE levels were found to be of diagnostic significance in differentiating between: (a) pemphigus and bullous pemphigoid, and (b) patients with allergic bronchopulmonary aspergillosis and those with aspergilloma or other chronic lung conditions demonstrating precipitins to aspergillus.

AI 11,202-03, Ishizaka, K., Johns Hopkins. In studies to elucidate the regulatory mechanisms of IgE antibody, T cell activity factors were identified and found to be different for IgE and IgE enhancement responses. Anti IgE responses were also effected in an experimental model offering possible leads to explore methods for prevention or suppression of antibody responses as a fundamental aspect of treatment for allergic disease.

AI 11,759-01, Patterson, R., Northwestern University. Methods effecting states of reaginic hypersensitivity by inducing IgE responses to naturally occurring antigens in dogs and primates have been established. Studies utilizing these unique animal models of human allergic disease have demonstrated that: (1) the pulmonary resistance functional test is one of the most sensitive measures of immunologic response of the airways, (2) frequency-compliance change is probably the earliest sign of response, (3) any differences in processing ragweed antigen between normals and allergics did not reside in pulmonary alveolar cells, and (4) naturally occurring immediate hypersensitivity of bronchial target organs can be effected by inhalational exposure.

B. Clinical Immunology & Immunopathology (SEP)

The program in clinical immunology and immunopathology has been concerned with investigations of etiologic factors, pathogenetic mechanisms, detection of critical diagnostic measures and approaches to effective prevention, control of immunologic deviations, abnormalities and dysfunctions. Its thrust continues to be directed to application of data generated by immunobiologic research to an understanding of causative factors and corresponding management of immune-related diseases. Major delineated disease problems upon which especial attention has been currently focused have been autoimmune disorders, immune complex formation and deposition, transplantation phenomena, immune deficiency states, action of pharmacologic and chemical mediators of inflammation and immunotherapeutic modalities. This program has been supported at a level of $4,705,000.

AI 11,313-03, McDevitt, H.O., Stanford University (1) The nature of circulating DNA, the biological reactivity of antibody to DNA, or the presence or absence of reaction inhibitor may be more important in explaining the character and localization of tissue damage in systemic lupus erythematosus than the serological reaction per se in systemic lupus erythematosus. (2) In the search for histocompatibility linked immune response genes in man considerable polymorphism in response to immunization to diphtheria and tetanus toxoids have been demonstrated. (3) Although spontaneously occurring and radiation induced forms of Graves disease appear to be similar the study of genetic markers, W28 and HL-A5, suggest that they have distinct pathogenetic mechanisms selective against genetically different hosts. Such distinction may be applied in seeking correlations between genetic markers and prognosis or response to therapy in differentially susceptible individuals.

AI 07854-08, Rothberg, R.M., University of Washington. It has been determined that while oral immunization in premature infants is possible, there is a threshold for immune response to oral challenge to soluble protein antigen in infants weighing 1500 grams or less.

AI 09145-14, Fudenberg, H.H., University of California, San Francisco. Adult onset common variable immune deficiency has been shown to be genetically determined based on tests of DNA and RNA synthesis; heterogeneity demonstrated by familial GM marker imbalance. A defect in monocyte Ig receptors has been shown in one of two distinct forms of Wiskott-Aldrich syndrome.

AI 09738-04A1, Lamm, M.D., New York University. Immunoglobulin A has been isolated from human colostrum and purified, and a major part of chemical characterization of the secretory component has been accomplished.

AI 10,343-04, Hirschhorn, R., New York University. In genetic absence of red blood cell adenosine deaminase (ADA) giving rise to combined humoral and cellular immunodeficiency, the absence of five different tissue specific isoenzymes of ADA has been found and the feasibility of accurate prenatal diagnosis of this condition ascertained. In the study of this same disorder, Meuwissen, R.H., AI 11,717-02, New York State Department of Health, has established feasible methods for the differentiation of combined immunodeficiency disease by the presence or absence of ADA matching histopathologic criteria and has developed a blood spot screening test for newborns.

AI 10,591-03, Zweiman, B., University of Pennsylvania. An autoimmune nature for multiple sclerosis is additionally suggested by the finding of elevated levels of myelin-binding gamma globulin in sera of patients. In turn leads for the evaluation of azothiaprine as an immunosuppressive agent in the treatment of this neurological disease have been obtained.

AI-10,600-04, Root, R.K., University of Pennsylvania. A comprehensive screening program of granulocytic functions has been conducted in patients with increased susceptibility to infection leading to the identification of inherited defects of host defense and cellular problems in patients with diabetes and uremia. Results of studies on the hydrogen peroxide related granulocyte functions offers leads for correcting metabolic and bactericidal abnormalities in chronic granulomatous disease of childhood.

AI-11,280-02, Johnson, A.M., University of North Carolina. Studies of the relationship of α_1 antitrypsin deficiency to atopic diseases have indicated that specific protease inhibitor phenotypes occur more frequently in children with asthma and chronic bronchitis and that decreases in serum concentrations occur during acute attacks. These findings thus suggest that excessive antiprotease utilization can serve as a causative factor in these conditions.

AI 11,526-02, Ward, P.A., University of Connecticut. Increased levels of a serum inactivator of chemotactic factor have been demonstrated in Hodgkin's Disease indicating a mechanism for the inability of patients with this disease to express inflammatory responses based upon a defect in a natural regulator of inflammation.

AI-11,843-02, Good, R.A., Sloan-Kettering Institute. (1) In a study of skin diseases the finding of involvement of complement in the development of lesions of pemphigus and pemphigoid suggests its autoimmune nature. (2) Two distinct forms of congenital neutropenia have been identified; only one showing a defect in marrow cells. (3) Specific serum complement deficiencies have been demonstrated in lupus erythematosus, necrotizing arteritis and progressive renal diseases indicating a mechanism for severe susceptibility to infection in these disorders. (4) Both T and B cell immunodeficiencies and effects of cytotoxic drugs and irradiation have been implicated as contributing factors in the development of *Pneumocystis carinii* pneumonia. (5) Evidence has been given for the non-specific action of transfer factor.

AI 12,049-02, Jordan, R.E., Mayo Clinic. Studies concerned with specific skin diseases point to their pathogenesis on an immunologic basis. Findings related to activation of complement pathways, deposition of complement components, properdin and immune complexes in affected tissue and immunologic assays of blister fluid implicate herpes gestationis, pemphigus vulgaris and bullous pemphigoid in this category.

AI 10,334-04, Andres, G. SUNY at Buffalo. Evidence has been obtained that interstitial nephritis may be produced by the same mechanisms responsible for glomerular kidney diseases; the role of antibodies to tubular basement membrane demonstrated. The association of circulating antigen-antibody complexes with active systemic lupus erythematosus was also shown.

AI 07007-10, Dixon, F.J., Scripps Clinic and Research Foundation. (1) Isolates of oncornavirus-like activities from placentas of women with systemic lupus erythematosus suggest their possible involvement in human immunologic disease. (2) A high proportion of patients with IgG multiple myeloma were found to exhibit a defect in polymorphonuclear leucocyte function. (3) Patients with anti-glomerular basement membrane type nephritis (GBM) and immune complex type demonstrated depositions of specific complement components in the kidney indicating the responsible pathways of activation. (4) Anti GBM antibodies were found to be directed toward the non-collagenous portion of the GBM, the pathogenicity of antitubular basement membrane antibodies in renal damage was observed, and the occurrence of extraglomerular deposits was found to correlate with tubulo-interstitial injury in patients with systemic lupus erythematosus. (5) The presence of vasoactive materials was shown to enhance the renal deposition of circulating immune complexes but that the administration of excess antigen can dissolve glomerular immune complex deposits and prevents the formation of additional deposits by terminating immune responses in chronic serum sickness. (6) An extremely sensitive method for the detection of immune complexes in sera from patients with glomerulonephritis has been developed with the use of cultured Raji cells based on their receptors for IgG and complement components in the absence of an ability to produce Ig themselves. (7) Specific viruses (LCM and LDV) by infecting the lymphoreticular system not only alter the function of lymphoid cells but also by associated nonspecific activation of macrophages effect increased host resistance.

1. Transplantation

Future advances in organ transplantation in clinical medicine will depend upon our knowledge of relevant immune mechanisms. Accordingly, attention has been directed to investigations concerned with transplantation and rejection mechanisms. Basic to this goal is an understanding of the genetics of immunologic phenomena. Thus, included in this program are studies on histocompatibility antigens in immune regulatory mechanisms and disorders and products of the Ir gene reaction. Initial research in this area focused on areas dependent upon HL-A tissue typing. However, more recent studies have demonstrated that while this approach may explain events of rejection, the mixed leucocyte culture reaction (LCR) is a more reliable indicator of the first stage, i.e. recognition phenomena. Thus, investigations based on LCR are receiving increasing attention. However, tissue typing approaches have given leads to a new area of investigation, the relationship of HL-A types to specific disease entities as a function of genetic predisposition. This program has been supported at a level of $2,329,000.

AI 10,658-03, Rapaport, F.T., New York University. Development of a highly inbred line of dogs has favored studies applicable to problems of bone marrow, skin and kidney grafts in man. It was found that in spite of linkage disequilibrium between serologically (SD) and mixed leucocyte culture detectable phenotypes (LD) the products of LD locus exert as great if not greater influence upon the fate of allografts than SD antigens.

AI 10,678-04, Billingham, R.E., University of Texas Southwestern Medical School. Maternal leucocytes were detected in the circulation of infants in animal models as a result of absorption from milk. Such naturally transmitted cells were shown to be capable of: (1) adoptively immunizing their recipients causing them to attain immunologic maturity precociously, (2) specifically impairing their reactivity against allografts of the same alien genetic origin as the milk by inducing tolerance, and (3) prejudicing the host's well being or even its survival through graft-vs-host reactivity on the part of the immunocyte moiety. Questions are thereby posed regarding the immunologic significance of leucocytes present in human milk and effects by infant feeding.

(4) The spleen has been found to serve a function of immunologic privilege and the development and full expression of systemic cell-mediated immunity may be interfered with if an animal's initial immunologic encounter occurs within that organ. (5) In considering need for means of attenuating and aborting severe graft-vs-host (GVH) reactions following bone marrow transplantation attention has been directed to excessive autoantibody production resulting from the allogenic effect of T lymphocytes attacking host B cells and stimulating quiescent self-reactive clones.

AI 11,051-02, Barker, C.F., University of Pennsylvania. (1) Studies of models for blood vessel transplants in which endothelial repopulation serves as the antigenic stimulus suggest that human venous allografts may be feasibly accomplished by slowing the cellular regeneration factor. (2) Studies of models of transplantation of pancreatic islets for possible replacement treatment of diabetes indicate minimization of rejection if islets are embolized into the liver; thus suggesting the liver to be a semi-privileged site for the pancreatic tissue.

AI 06320-10, Russell, P., Massachusetts General Hospital. (1) In the study of problems related to kidney transplantation: (a) antithymocyte globulin infusion in high dosage regimes has been efficacious in conjunction with conventional immunosuppression, (b) the use of frozen blood has been of value in avoiding sensitization to the alloantigens present on leucocytes. (2) Successes have been achieved in treating potentially lethal burns with fresh skin from relatively histocompatible family members by immunosuppression with azothiaprine. (3) Bone marrow infusions from HL-A sibling doners for treatment of aplastic anemia gave evidence of engraftment and clinical improvement.

AI 06918-10, Winn, H.J., Massachusetts General Hospital. (1) Hemorrhage and platelet thrombi were shown to be consequences rather than causes of the immunologically mediated tissue damage of transplantation rejection. (2) Development of insensitivity of skin grafts has been shown to be not related to immunosuppression, and sensitive grafts if retransplanted again develop initial resistance. Local changes in the graft are important with newly regenerated small blood vessels resistant to a variety of inflammatory stimuli regardless of specificity. (3) Ig^{M} class antibodies have no deleterious effect on grafts and may in fact block the effects of other classes of antibodies. This IgM response is thymic dependent and not altered by allogeneic lymphocytes. (4) Genetic analysis has shown that lung survival of grafts is dominant to short survival in animal models immunosuppressed by thymectomy and antilymphocyte globulin.

AI 08074-07, Stuart, F.P., University of Chicago. (1) Passive immunization may prolong renal allograft survival by delaying the development of cell mediated immunity for sufficient time to allow production in vivo of serum blocking factors to prevent rejection; such factors reflected by rising antibody titers in the graft recipient. (2) Antireceptor antibody may inhibit cell-mediated responses as well as antibody responses to histocompatibility antigens and play a role in the regulation of immune responses to such antigens. (3) The presence of induced anti-alloantibody can function as anti-receptor antibody and plays an important correlating role in resistance of hybrids to graft-vs-host disease. (4) Delay of transplantation until anti-receptor antibody (ARA) reached peak titers led to optimal suppression of renal allograft rejection, suggesting that ARA can play a role in suppressing host responsiveness by blocking the host lymphoid cell receptors for histocompatibility antigens of the graft.

AI 02425-12, Thomas, E.D., University of Washington. Advances in procedures employing bone marrow transplantation have been recorded. Normal marrow from identical twin donors can survive in patients with aplastic anemia and can induce frequent and enduring remissions in endstage patients with acute leukemia. In case of HL-A matched donor-recipient sibling pairs, non reactive in mixed leucocyte culture tests, treatment regimes involving radiation or antithymocyte globulin with bone marrow transplantations have been developed and shown to be capable of achieving long term survival in patients with even endstage acute myelogenous and lymphatic leukemia.

C. Immunobiology & Immunochemistry

Research supported within these program areas is directed toward acquiring a complete understanding of immune response mechanisms at their basic cellular and molecular levels. By design, such broadly based programs cross traditional disciplinary lines of biology and chemistry and encompass anatomic, genetic, physiologic, pharmacologic, microbial, and developmental biology and organic, physical, and biological chemistry. Although these programs have the acquisition of fundamental knowledge as their overall objective, they are complementary to the Asthma and Allergic Disease and Clinical Immunology Programs which the Institute supports in its efforts to develop appropriate prophylactic, diagnostic, and therapeutic approaches to the allergic, infectious, immunologic, and neoplastic diseases of man.

The scope of these programs is purposely broad and includes studies of immunologic responsiveness in various hosts as well as of the extensive variety of antigenic stimuli which initiate immune responses. Thus, phylogenetic studies concerned with the development of immune response capabilities in a range of vertebrate hosts, ontogenic studies of immunologic responsiveness within an individual species, genetic controls of responsiveness in individual hosts, interactions of immunologically competent cell populations within the host, as well as physico-chemical studies of antigens and their homologous antibodies, all are appropriate topical areas within these programs.

The extent of activity in these programs is reflected by the number of research awards. During FY 1975, these programs supported 189 active and 38 newly-awarded individual research grants, 4 active program projects, and 13 active and 5 new career development awards. Also included within these program areas were 13 training awards which are in final phase-out stages and 12 active individual fellowship awards to which approximately 60 more are expected to be added during the current fiscal year.

Support for the research activities within these program areas is grouped in two circumscribed Special Emphasis Programs and in three general areas of interest.

1. Lymphocyte Biology (SEP)

   The ultimate goal of this Program is the complete knowledge of the life history of immunocompetent cells and of the genetic and phenotypic factors that determine their fate and function _in vivo_ and _in vitro_. The knowledge gained from this effort should provide a complete understanding, and the means for control, of every facet of the immune response. At present, this program supports four active program projects. Each is a multi-disciplinary research center into which has been integrated the technical expertise of cell culturists, geneticists, cellular immunologists, and immunochemists. Their combined efforts are designed to expand our knowledge of the morphological and functional heterogeneity of lymphocyte populations and to develop the capability for identification and selection of lymphocyte sub-populations with specific immune reactivity or antigenic composition, for hybridization of such sub-populations, and for selective production of specific lymphocyte products. Practical application of this knowledge would lead to the use of selected, cloned, lymphocytic cells and their products for the clinical care or reconstitution of immunologically deficient individuals, to alleviate allergic states, to provide specific resistance to microbial infections, and to cope with neoplasia.

   Research activity within the four projects comprising this Program has continued to be intense and productive. The multi-disciplinary approach has proven to be workable and highly effective and holds great promise to significantly advance knowledge and understanding of this key cellular population.

AI 10,702-04 M. D. Scharff (Albert Einstein College of Medicine)

The techniques of cell biology and somatic cell genetics are being applied for study of cloned, mutant, and wild type cell cultures to gain a better understanding of the immune response and the molecules responsible for various immunologic phenomena. Characterization of an immunoglobulin produced by a cloned, mutant, culture of lymphocytes is under way and indicates that the mutation is being expressed in the C terminal end of the heavy chain. Interest in this product has centered on the nature of the mutant lesion and the mechanism, either transcription or translation, of synthesis which is affected. The structural and biochemical properties of the H-2K and H-2D alloantigens of mouse lymphocytes are being examined as are those of the products of the I region, the so-called Ia antigens, to determine the interactions of these genetic factors in controlling the synthesis of immunoglobulins and mediator substances. Fractionation of these materials is in progress to prepare homogeneous materials for subsequent amino acid, N-terminal, and partial sequence analysis. Fusion of phenotypically similar cells also is being employed to aid in the genetic and biochemical analysis of mutant cell cultures. Efforts are being made to develop homogeneous cultures of T and B lymphocytes and macrophages for studies of their complex interactions and the products of their interactions which influence many cell-mediated immune reactions. An antibody-dependent cell-mediated cytotoxicity of viral infected cells has been demonstrated for the first time as a result of this work.

AI 11,378-02 G. M. Edelman (The Rockefeller University)

The number, specificity, and functional properties of antigen-binding cells in fetal and adult mice are being determined to achieve a complete description of the clonal selection process and to clarify the mechanisms by which antibody diversity is generated. The results of studies on the maturation of the immune response in mice indicate that a large number of different, specific antigen-binding cells arise early in ontogeny of the immune response system and that the range of avidities of cells which bind a specific antigen does not change greatly during development and maturation to adult life. The results of ultrastructural and chemical studies of cellular receptors indicate that the modulation of receptor mobility occurs at the level of individual receptors on the cell surface and are expected to lead to the identification and isolation of the microtubular structures which are responsible for receptor modulation and cellular stimulation by antigens and mitogens. A chemical characterization and structural studies of the major histocompatibility antigens of the mouse (H-2) and man (HL-A), as well as of β_2-microglobulin and α_2-macroglobulin, are being continued of these molecules which serve important functions on the surfaces of lymphoid cells. Similar studies of various mitogens have been initiated to complement membrane receptor studies and are expected to increase understanding of the molecular basis of signalling events at the lymphocyte surface which trigger the differentiative and proliferative events of immunologic significance.

AI 11,576-03 F. H. Bach (Univ. of Wisconsin School of Medicine)

Genetic studies of *in vitro* lymphocyte responses have demonstrated that surface molecules controlled by the LD and SD loci interact and mediate the generation of cytotoxic cells. Emphasis also is being given to the development of methods for rapid definition of HL-A LD alleles; the preliminary results of such a test suggest that the LD phenotype of any individual can be determined rapidly by testing his cells against a battery of pre-sensitized cells. This system appears to permit the definition of any LD alleles that differ by a single haplotype and to obviate the problem of locating homozygous typing cells. In other studies, H-2 antigens have been identified as the prime cytotoxic targets in the xenogeneic mixed lymphocytes culture system and, in allogeneic combinations, the SD antigens are the targets. The results of ontogenic studies suggest that cell populations responsible for the proliferative response are present at birth and increase somewhat during the early postnatal period but that cells functional in the cytotoxic response are not detectable until after the first postnatal week. In studies of immunodeficiency disorders, preliminary evidence has been obtained which indicates that certain subpopulations of lymphocytes, "null" cells, can be converted to active T cells by treatment with cyclic nucleotides, purine bases, transfer factor, or thymosin.

AI 11851-02 J. W. Uhr (Univ. of Texas Southwestern Med. School)

Studies of molecular structures on the surface of murine B cells have led to the identification of two immunoglobulin classes; one has a heavy chain which has been identified as μ type and the other, smaller in size than μ chain, has properties similar to those of human δ chain and may represent the murine counterpart of human IgD. It has been established that newborn mice have only IgM on the surface of their splenic lymphocytes; the IgD-like molecule appears later after birth and subsequently becomes the predominant cell surface Ig. These results indicate that surface IgD is acquired by cells initially bearing only IgM during their differentiation in the spleen and that a large lymphocyte with surface IgM is probably the precursor of small lymphocytes with both surface IgM and IgD. Other studies have indicated that TL and H-2 antigens have structural homology and may have evolved from the same primordial gene. Evidence for a functional linkage between H-2 and TL antigens also has been obtained; when antigen modulation occurs during the phenotypic loss of TL antigen, there is a proportionate increase in H-2D antigens. It would appear from such data that the disappearance of TL antigen and marked increase in surface density of H-2 antigen in differentiating thymocytes can be considered operationally analogous to the differentiation of B lymphocytes in which there is a switch in expression of surface Ig from μ chain to other heavy chain classes.

2. Selective Immunologic Activation (SEP)

The stated objective of this Program is achievement of the capability for selective manipulation of the immune response in order to purposely direct host responses to either cell-mediated immunity or to humoral antibody production. Because it now has been established that the immunologically-competent lymphocytes which are responsible for humoral and cellular immune responses, the bone-marrow (B) and thymus-dependent (T) lymphocytes respectively, interact to either inhibit or potentiate each other, B- and T-cell interactions must be better understood to effectively control the nature of the immune response. To this end, the program is supporting 38 individual research projects concerned with the nature of the interactions among these cells, their differential susceptibility to modifying agents, and the influence of variations in the molecular features, dose, and route of antigenic stimulation on the responses of these individual cell types. The content of this program was intended to complement that of the Lymphocyte Biology Program and, as such, also supports individual projects concerned with lymphocyte recognition systems, activation, and suppression mechanisms and includes relevant studies of processes leading to lymphocyte differentiation and maturation and their production of biologically-active substances which mediate immune reactions. The development of an immune response control capability would have direct clinical application in providing more effective means for prophylaxis and therapy of infectious, allergic, and autoimmune diseases, for prevention of transplant rejection, and for management of neoplasia and certain aging syndromes.

Interest and activity within this topical area remain high and several approaches are being employed to develop an understanding of the mechanisms which initiate and selectively control humoral and cellular immune responses.

AI 04,197-13 F. W. Fitch (Univ. of Chicago Medical School)

The surface antigen receptors of lymphocytes destined to form antibody appear to have the same or similar characteristics of the immunoglobulin which these cells subsequently synthesize and release. Antibody against these receptors has been found to inhibit humoral antibody formation by B-cells and it inhibits the grant-versus-host and mixed lymphocyte culture reactivity of T-cells.

AI 09867-05 A. J. Crowle (Univ. of Colorado Medical School)

Sera from mice whose delayed-type, cell-mediated, hypersensitive reactivity was abolished by multiple injections of homologous antigen contain humoral antibodies which prohibit the induction of cell-mediated hypersensitivity when injected into other mice prior to immunization with the same antigen. This "contrasensitizing" antibody acts early in the induction of delayed hypersensitivity, perhaps at the stage of antigen processing by macrophages or in the early interaction of T-cells with other T-cells or with B-cells.

AI 09,920-05 B. Benacerraf (Harvard Medical School)

The "helper" function of T-cells which enhances B-cell immunoglobulin synthesis can be abolished in guinea pigs by injection of unrelated antigens. This alteration of the immune response appears to be attributed to "antigen competition" and may involve the production of soluble mediator substances by T lymphocytes.

AI 10,225-03 D. C. Benjamin (Univ. of Virginia School of Med.)

Rabbits immunologically tolerant to a protein antigen were found to have normally functional B-cells. The tolerance lesion which prohibits immunoglobulin synthesis was localized at the helper T-cell level; adoptive transfer of normal rabbit thymocytes to tolerant animals led to restoration to immunologic responsiveness to the tolerogen.

AI 10,242-15 D. A. Rowley (Univ. of Chicago Medical School)

Anti-receptor antibody has been demonstrated to reversibly block B-cell responses in adult mice without reducing the size of the responding cell clones. In neonatal mice, however, it depletes the clone of specifically responding cells and the animals remain unresponsive even as adults. These findings indicate that the response to an antigen is potentially regulated by both antibody to the antigen and antibody directed against the lymphocyte surface receptors for that antigen.

AI 11,945-02 S. B. Salvin (Univ. of Pittsburgh School of Med.)

Treatment of hypersensitive guinea pigs with varying doses of cyclophosphamide led to a graded reduction of antibody production by B-cells and a concomitant extension of delayed-type hypersensitive reactivity. This observation suggests that suppressor B-cells might regulate the activity of T-cells in the expression of delayed hypersensitivity skin reactions.

D. Acquisition of New Knowledge

The Immunobiology and Immunochemistry programs also support research in three general categories areas which complement the Special Emphasis Programs but are sufficiently distinct in content and objectives to be identified separately.

Research support in the Immunochemistry category centers on chemical approaches to immunologic reactants and reactions. Studies of the chemical composition and structure of immunoglobulins and antigens, as well as of the mechanisms and kinetics of antigen-antibody reactions, form the bulk of the activity in this category. However, there also is an active interest and moderate degree of activity in chemical studies of complement and its components. Research in the newly-developing field of immunopharmacology, which includes drugs, hormones, and other endogenous substances that influence or participate in the immune response, also is supported within this general area.

AI 05,664-11 J. W. Goodman (Univ. of California - San Francisco)

The results of studies with synthetic antigens have indicated an association between the steric availability of antigenic determinants and the helper function of immunocompetent lymphocytes. It has been demonstrated that at least two determinants on an antigen molecule must be accessible on the lymphocyte surface in order to obtain an antibody response; this observation strengthens the plausibility of antigen-bridging models of cell cooperation.

AI 06,860-09 B. F. Erlanger (Columbia University)

Antibodies to nucleosides and to nucleotide sequences have been prepared and are being employed as tools to study biochemical and biological systems that are mediated or controlled by nucleic acids. They have proven to be valuable for studies of the organization and architecture of human metaphase chromosomes, to detect chromosomal abnormalities, to understand the mechanisms of genetic diseases, and to increase knowledge of protein-nucleic acid interactions which play important regulatory roles in biological processes.

AI 09,341-05 L. T. Rosenberg (Stanford Univ. Medical School)

Genetic variants in the complement system of the mouse are being used to assess the role of complement in infection and in hypersensitive manifestations of the immune response. With such variant mouse strains, evidence has been obtained which indicates that C'_5 negative animals have a greater survival rate when acutely infected with toxoplasma, although preliminary data suggest that the presence or absence of C'_5 has little influence on infection with mouse-adapted malaria parasites.

Interest in the *Immunogenetics* category also is quite broad and ranges from studies of the phylogenetic and ontogenic development of the immune response to studies of genetic controls of immune responsiveness in individual hosts, inbred strains of animals within a species, and in a variety of species. Much of the information gained from such studies is applicable not only to the Lymphocyte Biology and Selective Immunologic Activation Special Emphasis Programs, but also to the Clinical Immunology Programs, because recent evidence suggests that susceptibility to certain diseases may be determined genetically. Also, a considerable portion of the work supported in this category is concerned with genetic influences on histocompatibility and has relevance to transplantation immunology.

AI 06,525-10 J. H. Stimpfling (McLaughlin Research Institute)

Approximately 55 strains of mice, which are congeneic with $C_{57}B_1/_{10}$ but are recombinants within the H-2 locus, have been developed and are being maintained for extensive studies of the serological complexity of the H-2 locus and of its role in regulation of immune responses.

AI 09,533-03 B. G. Sanders (University of Texas)

An inbred strain of chickens, for which data on alloantigen inheritance are available for four generations, is being maintained for studies of immunoglobulin complexity and structure to develop comparative data on the origin and evolution of the immune response.

AI 10,679-03 M. Borysenko (Tufts Univ. School of Medicine)

Reptiles, representing the closest ancestors of endothermic vertebrates, are being studied to compare their immunologic functions with those of animal classes evolutionarily above and below them. Graft-versus-host disease has been effectively induced in the snapping turtle and is maximized by high genetic disparity between the host and the allogeneic spleen cell donor. The results of early studies on allograft rejection in the South American lungfish, a link between bony fishes and amphibians, indicate that it also rejects skin allografts in a chronic fashion.

AI 10,961-04 D. B. Wilson (University of Pennsylvania)

Selected populations of rat T-lymphocytes which have specific immunologic reactivity to chosen histocompatibility alloantigens have been found among the cellular products of mixed lymphocyte cultures. Evidence indicates that such lymphocytes are not terminally differentiated end cells but have the capacity for subsequent reactivity both in graft-versus-host and mixed lymphocyte reactions.

The Cellular Immunology and Immunity category is focused on cellular aspects of the immune response and therefore, like other general categories, also serves to complement the research supported by Special Emphasis Programs. Research supported within this category is concerned with the cellular events associated with the immune response, including interactions of T-lymphocytes with other T-lymphocytes, as well as with B-lymphocytes and other leukocytes, and the phenomenon of immunologic tolerance. The cellular mechanisms involved in the induction, maintenance, expression, and pathophysiology of delayed-type hypersensitive immune reactivity also are included in this category, as are studies of the lymphocyte mediator substances associated with delayed hypersensitivity. The degree of activity in this area is relatively high mainly because of the recognition that T-lymphocytes are involved in a broad spectrum of infectious diseases and neoplasia and that their mediator substances may have considerable therapeutic value.

AI 07,118-09 B. R. Bloom (Albert Einstein College of Medicine)

A highly sensitive isotopic labeling technique has been developed for measuring small amounts of protein secreted by immune lymphocytes on *in vitro* stimulation. At least 50 proteins, many of which are not present in cytoplasmic extracts but are clearly synthesized for secretion, have been identified by this method.

AI 07,685-10 J. R. David (Robert B. Brigham Hospital)

Mediator substances produced by lymphocytes from hypersensitive donors are being isolated and characterized and their *in vivo* and *in vitro* biological functions are being evaluated. Normal guinea pig macrophages, which were activated by mediator substances, exhibited increased cell adherence, spreading, phagocytosis, and glucose oxidation. The enhanced bacteriostasis of *Listeria monocytogenes* also observed in such activated macrophages implies an important role for mediator substances in immunity to intracellular infections.

AI 09,460-05 G. A. Granger (Univ. of California - Irvine)

In vitro studies of the activation of murine and human lymphocytes to become cytotoxic effector cells and release soluble lymphocytotoxin have led to the separation and identification of the cell types involved and the steps which occur during activation, lymphocytotoxin secretion, and acquisition of effector functions by lymphocytes.

AI 10,351-05 R. J. North (Trudeau Institute)

An immunizing infection of mice with *Listeria monocytogenes* has been found to generate active T-cells, delayed hypersensitive reactivity, and systemic activation of macrophages which is lost soon after the infection has been resolved. Immunity to reinfection was shown to require a secondary immune response in order to generate a new population of mediator-producing T-cells and to reactivate effector macrophages.

II. BACTERIAL AND MYCOTIC DISEASES BRANCH

The approximate level of research support in bacterial and mycotic diseases:

Type of Grant	Number	Amount
Research Grants 1/	226	$10,734,461
Training Grants	29	1,666,153
Fellowships	19	218,800

Summary

Research progress in infectious diseases of bacterial and fungal origin clearly interfaces with the basic disciplines of microbiology, cell biology, and biochemistry. As these sciences have broadened and expanded, the fundamental knowledge thus gained has enlarged the more applied clinical and therapeutic advances made in infectious diseases research. The Bacterial and Mycotic Diseases Branch has supported, in addition to categorical infectious diseases research, programs dealing with highly-relevant studies in the basic biomedical sciences. Studies in cell biology, for example, are particularly appropriate to furthering our understanding of cellular immunity mechanisms as applied to problems of hospital-associated infections.

In addition to supporting an array of grants covering the broad spectrum of biomedical research involved in infectious diseases, the Branch also supports research in three categorical Special Emphasis Programs --

Biology of Venereal Diseases, Hospital-Associated Infections, and Streptococcal Diseases and Sequelae. The first two programs have shown a continued growth, indicative of an active sustained interest with the biomedical community. The Dollar Allocation of Program Area (DAPA) mechanism of funding has played an important role in the development of these Special Emphasis programs.

Two trends that will be closely observed in the future are an expanding interest in research on diarrheal diseases, as well as research leading to the development of ribosomal vaccines for a number of different diseases.

A. Biology of Venereal Diseases (SEP)

The problem of sexually-transmitted diseases, from a public health standpoint, continues unchecked, although there appears to be a small decline in the reported cases of primary syphilis, As for the sexually-transmitted viral diseases, the problem of genital herpes infections appears to be increasing. Evidence now being uncovered by research workers in England also suggests that infectious hepatitis (Hep A) may

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1/ Includes 10 career awards ($324,535).

be transmitted by sexual contact. It is evident that much research remains to be done in the field of sexually-transmitted diseases before control measures will be fully effective in modifying the current wide-spread problem. The Special Emphasis Program on Biology of Venereal Diseases has maintained a self-sustaining momentum in its continuing growth. A very great interest in research in this area has been engendered within the biomedical community.

The program currently supports a total of 54 grants and fellowships, including three large program projects, individual research grants, individual postdoctoral fellowships and institutional fellowships. A total of 20 individuals are now undergoing training with both clinical and research orientation in venereal diseases. By the end of FY 1975 the total program will be funded at a level of approximately $3.0 million. A fourth program project will be presented to the NAAIDC in June of 1975 for final action.

Meetings:

The NIAID has contracted to support the First Symposium on the Biology of the Parasitic Spirochetes, to be held at the University of Minnesota in June 1975. This meeting is the first of its kind to be devoted to all aspects of the spirochetal organisms, but with emphasis on *Treponema pallidum*. This organism, the causal agent of syphilis, has not engendered the same research interest as has the gonococcus, due in large measure to the greater difficulty of working with the treponemes; the treponemes have not yet been grown *in vitro*. There is also a renewed research effort by virologists, who are beginning to show interest in both the herpes type II and cytomegalovirus infections as they relate to the venereal disease problem.

Highlights of the Special Emphasis Program on Biology of Venereal Diseases follow:

AI-10982-02 C. D. Cox (University of Massachusetts)

This investigator, studying the physiology of *T. pallidum*, has shown that the virulent organism consumes O_2 at a rate similar to the known aerobic spirochete, Leptospira. This O_2 uptake is not dependent on the presence of reduced extracellular ions, and is inhibited by micromolar levels of NaCN, azide, and amytol. This indicates the presence of a functioning cytochrome oxidase system and functioning electron transport system, as in other aerobes. Contrary to the view that has long been held, the results of this intriguing study strongly indicate that *T. pallidum* is an aerobic organism rather than an anaerobic one.

AI-11283-02 J. B. Baseman (University of North Carolina)

Dr. Baseman has developed a technique for obtaining purified treponemes, free of extraneous matter, for biochemical analyses by velocity sedimentation. He has also found that virulent treponemes incorporate radiolabeled amino acids and thus are not defective in their ability to synthesize protein during *in vitro* incubation. These data will help clarify the spirochete's metabolic activities and may lead to a means of *in vitro* cultivation of this organism.

AI-11305-02 D. M. Musher (Baylor College of Medicine)

In his interesting studies on the cell-mediated immune response of syphilis, Dr. Musher's earlier hypothesis has now been confirmed. His data show that in an infected rabbit model system there is indeed a generalized suppression of the lymphocyte response during syphilitic infection. It is believed that this is the first such direct experimental evidence reported on this phenomenon.

AI-12192-01 K. K. Holmes (University of Washington)

This group has had a long-standing interest in the problem of nongonococcal urethritis (NGU), one of the most common sexually-transmitted diseases, at least as reported in the Seattle area. The investigators report that 50% of the NGU cases in Seattle were associated with one of the chlamydia agents, *Chlamydia trachomatis*. The *C. trachomatis*-positive cases responded quickly to therapy with sulfonamides.

The third most common venereal disease infection seen by the Principal Investigator, after gonorrhea and NGU, is herpes virus infection of the genital tract, in both males and females. These infections are a major problem because, as with most herpes infection, there is a very high rate of periodic reoccurrence following the initial infection. These infections, furthermore, are difficult to treat, as no specific antiviral therapy is yet available.

Gonococcal arthritis has also been studied by this group of investigators. This syndrome, found primarily in infected females as a sequelae of gonococcal infection, was found to be caused by a single immuno-type of *N. gonorrhea*. This preliminary report regarding a single immunospecific gonococcal agent may have important implications in further study of the gonococcus and its sequelae in the infected female.

AI-10669-03 R. B. Roberts (Cornell University)

This project focuses on studies on the interaction between polymorphonuclear leukocytes (PMNs) and the gonococcus. It was shown that the gonococcus (GC), as a stable colony type, could be readily obtained from the chorioallantoic cavity of the 10-day-old chick embryo. Log-phase virulent GC (types 1 and 2) resisted phagocytosis by PMNs, whereas the same organisms, in the stationary phase of their growth cycle, were ingested and rapidly killed. Log-phase avirulent GC were ingested and killed, but only in the presence of fresh, normal serum. The virulent resistant GC organisms were shown to attach to the surface membrane of the PMNs and macrophages.

These virulent organisms have antiphagocytic surface factors. Attachment to the surface membrane of the phagocytic cells may be a unique property of the GC and may play an important roll in the patho-physiology of gonorrheal infections.

AI-11073-03 J. L. Swanson (University of Utah)

As with a number of other research projects on the gonococcus, the goal of this project is to understand the interactions of gonococci and eukaryotic cell surfaces. Dr. Swanson demonstrated by electron-microscope techniques that gonococci with pilated surfaces underwent ingestion by leukocytes and also that ingested pili emanated from leukocytes that had completely engulfed the bodies of the pilated GC. Pilated GC were also found to adhere avidly to human sperm cells; this attachment was markedly reduced in the presence of anti-pilus antiserum. Preliminary experiments indicate that the change from pilation to non-pilation of GC is most likely a mutational event. Studies also suggest that the pili enhance attachment of the GC to non-leukocytic eukaryotic cells.

B. Hospital-Associated Infections (HAI) (SEP)

Although definitive data are hard to come by, one study has indicated that approximately 330,000 cases of Gram-negative bacteremia occur annually, with about 100,000 deaths. Gram-negative infections are among the major problem areas, with fungal infections now being recognized as of increasing importance in HAI. This is obviously a problem of great magnitude, requiring a vigorous effort by NIAID in support of research to find means of countering the enormous costs in mortality and excess hospitalization charges.

The program as it is now constituted comprises 36 grants and four individual postdoctoral fellowships. There are, however, no institutional postdoctoral fellowships. No program project grants were reviewed in FY 1975, although several now are in the planning stages.

Meetings:

The summary of the NIAID workshop on Hospital-Associated Infections, which formed the basis for the development of this Council-approved Special Emphasis Program, was published in the Journal of Infectious Diseases in December 1974.

Some interesting findings by grantees are briefly described here:

AI-11116-03 W. R. McCabe (University Hospital, Boston)

The goal of this grant is to evaluate the feasibility of immunization against Gram-negative infections, utilizing antigens shared by most of the Gram-negative bacteria (GNB). Titers of antibody in patients to various types of antigens found in these bacteria were correlated with frequency of shock and death. A distinct correlation was seen between rising titers of antibody to Re antigen and a decreasing frequency of shock and death. Other data indicate that the IgG, but not the IgM, class of antibodies in the patients to the infecting organism exerts a protective influence to infections with GNB. The Re antigen that elicits these protective antibodies has considerable potential as a candidate vaccine material to counteract these serious infections in patients.

AI-00658-18A1 E. Neter (Children's Hospital, Buffalo)

This study also seeks to determine the effectiveness of a non-specific antigen, called Common Antigen (CA) of Gram-negative bacteria (GNB), as a potential for immunization of hospitalized patients suffering from GNB infection. Results indicate that not all GNB produce the CA -- it is apparently under genetic control; the best source is a rough mutant of *Salmonella minnesota*. It was found that the lipopolysaccharide structure of the microorganism is not a necessary part of the CA. Strong primary and secondary immune responses were elicited in rabbits to CA. A rapid gel-precipitate test was also developed to determine CA antibody levels in patients and normal subjects as potential vaccinees. Peritoneal or septicemic infections in patients apparently elicited higher CA titers than did enteric or urinary tract infections. These studies on the CA antigen also have considerable potential for development of a candidate vaccine.

AI-10108-04 A. Braude (University of California, San Diego)

This Principal Investigator and his research group have developed a novel approach for treating bacteremia caused by Gram-negative bacteria (GNB). They have produced an antiserum to a mutant strain of *E. coli*; this antiserum has proved effective against infections caused by a number of other Gram-negative organisms, including dangerous antibiotic-resistant strains of *Pseudomonas aeruginosa*. Controlled clinical studies in progress indicate a great therapeutic potential for this antiserum. The mutant *E. coli* strain used has an enzyme defect that has affected its ability to attach the O antigen side chains, leaving an exposed core. The antiserum is made against the exposed core of the *E. coli* -- this core region is identical in almost all Gram-negative microorganisms. This accounts for the broad range of activity of the antiserum against many of the other Gram-negative infecting organisms.

AI-06931-09 P. G. Quie (University of Minnesota)

This highly-productive group, studying host reactions to microorganisms in infectious disease, have made some important observations useful in expanding our understanding of host defense mechanisms. They have reported that storage of whole blood under usual conditions does not compromise chemotactic responsiveness of neutrophiles for at least 24 hours. Certain bacteriostatic antibiotics such as tetracycline and doxycycline decreased the phagocytic capacity of human neutrophiles. This suggests that antibiotics of this type may be contra-indicated in patients with abnormal neutrophile function. It was also found that patients with acute infections and with normal host response to them have hyperactive neutrophile chemotactic responses, as compared to uninfected controls. In contrast, patients with allergic manifestations or recurrent infections have depressed neutrophile responsiveness.

C. Disease Control Measures

1. Streptococcal Diseases and Sequelae (SEP)

This Special Emphasis Program has maintained a level research pace rather than showing an increasing momentum as seen with other Special Emphasis programs. Although scientific progress continues to be made, there is not, apparently, an influx of the newer young research workers with new ideas and techniques that are required for active development of a research area. The problem of streptococcal disease, especially the Group A streptococci and its grave sequelae, remains as a serious health problem, particularly regarding rheumatic valvular heart damage and acute glomerulonephritis.

These disease entities are most commonly seen in the lowest socioeconomic segments and minority groups within our population. Because the streptococcal diseases are no longer reportable to the Public Health Service, there are no firm figures with which to gauge the true magnitude of the problem.

Another aspect of increasing concern is that of the Group B streptococcal infections. These infections are seen with increasing frequency in hospital nurseries, and are among the leading causes of serious septicemia and death in neonates. Evidence is also accumulating that the Group B streptococci may be sexually-transmitted because they are frequently found within the birth canal of mothers of infected infants. More information here is sorely needed to clarify the role of this group of pathogens.

The current program on Streptococcal Diseases and Sequelae now comprises a total of 21 grants, including one program project and two postdoctoral fellowships. The small number of postdoctoral fellowships being supported may be indicative of the lack of interest in this field of biomedical research.

Some findings of interest from this Special Emphasis Program are described here:

AI-04342-13 E. N. Fox (University of Chicago)

The research by Dr. Fox and his colleagues has focused on development of a vaccine against streptococcal infections. The candidate vaccine now undergoing clinical trial is based on a material called M protein, isolated and purified from the streptococcal organism. This vaccine product has been tested in the form of a nasal spray, given to volunteers. The results indicate that a strong primary and secondary immune antibody response was induced in their sera. After challenge with live streptococcal organisms, these volunteers showed resistance to the establishment of streptococcal colonization in their upper respiratory tract. Further evidence indicated that high levels of secretory immunoglobulins (IgA) in the mucosal tissues affected adversely the adherence of the streptococcal organisms to epithelial cells in these tissues. The M protein vaccine preparation, therefore, appears to be a safe candidate vaccine material. Furthermore, it does not seem capable of "sensitizing" red blood cells; allergic or hypersensitivity reactions would therefore be minimal.

AI-09645-06 E. M. Ayoub (University of Florida)

This study is concerned with the pathogenesis of rheumatic valvular heart disease. The results of current studies indicate that the association of the streptococcal enzyme, collagenase, with strains of streptococci infecting the throat, but not the skin, represents a significant determinant of the invasiveness of the organism. This mechanism may be involved in the production of non-purulent complications following streptococcal infections.

AI-05844-10 W. J. Cromartie (Univeristy of North Carolina)

In his final report, this investigator described results of his work on Group A streptococcal cell walls. These contain toxic units that can act as mediators of tissue damage even after the the streptococcal cells are killed. An animal model system was developed which suggested that these toxic cell fragments may be involved in the pathogenesis of rheumatoid arthritis and rheumatic fever. Histological changes seen in the joints of rabbits following injection of the cell wall fragments were similar to those seen in early rheumatoid arthritis. Studies with labeled antibody demonstrated the streptococcal cell wall antigen in the synovial tissues; this antigen persisted within the animal's macrophages for about five weeks. The antigen presence correlated with the evolution of the inflammatory process. In the rat, a classical arthritis developed involving joints of the ankle, feet, knees, and wrists. The arthritis was associated with the presence of Group A cell wall fragments in these affected joints.

Other diseases such as cholera, tuberculosis and leprosy, not specifically identified as part of a Special Emphasis program, are nevertheless of interest and worthy of support as part of the Institute's responsibility for research on Disease Control Measures.

2. Cholera

AI-10466-03 L. J. Berry (University of Texas)

Dr. Berry has isolated and purified the flagella from *Vibrio cholera*. Two fractions were obtained, one of purified flagella and the other of purified material from the sheath covering the flagella. These fractions individually were able to protect adult mice and their progeny from challenge by virulent *Vibrio cholera* organisms. These findings, that the anti-flagella antibody is also protective, may open the way for use of flagellar material as part of a cholera vaccine. The data also suggest that these antibodies may interfere with the binding of vibrios to the intestinal mucosa, thus preventing colonization and the subsequent initiation of the disease.

3. Tuberculosis

AI-06578-10 P. T. Davidson (National Jewish Hospital, Denver)

Patients with drug-resistant mycobacterial disease apparently have some deficiency in their normal immune responses to infections. This is not well-understood; it may be in part related to altered immunologic mechanisms. These patients can be successfully treated by application of proper drug regimens and careful monitoring for drug toxicity. Fully susceptible tuberculosis strains have been made selectively resistant to single drugs such as viomycin and kanamycin. Preliminary studies show that the clinical practice of using these drugs, singly or in combination, may be contra-indicated. Patients with rifampicin-resistant infections can be successfully treated, if other drugs are available. The most pressing problem is in patients infected with the atypical tubercle bacilli, especially *M. kansasii* and *M. intracellulare*. The best results against these organisms were found when five or six drugs were used simultaneously, e.g., PAS, INH, rifampicin, ethambutol, and streptomycin. No other newer or more effective drugs against tuberculosis have been discovered to date.

4. Leprosy

AI-10094-04 W. E. Bullock (University of Kentucky)

In studies of human leprosy, this investigator found a significant increase in the numbers of B-lymphocytes in patients. His results also indicate that anergy -- or non-reactivity -- of the cellular immune system occurred in patients with lepromatous leprosy; this may be secondary to the destruction of T-lymphocytes. Dr. Bullock also reports that disturbances of recirculation of lymphocytes, in infected animals, is caused by extensive lymphocyte trapping, or sequestering, within lymphoid organs, especially the spleen. These findings may help to unravel the nature of the defects in cellular immunity imposed by chronic infections such as leprosy.

D. Acquisition of New Knowledge

A wide variety of investigator-initiated research projects are supported by this Branch. The grants highlighted in this section are primarily of a fundamental nature, and are not targeted or directed toward any specific Special program of the Institute. It is important to support these fundamental studies so as to broaden the information base for future biomedical discoveries.

AI-09366-05 T. A. Stamey (Stanford University)

This study focuses on the biologic differences between women susceptible or resistant to urinary infections. Previous data established that recurrent bacteriuria was preceded by the establishment of the pathogen on the vaginal introitus prior to the occurrence of bacteriuria. Women susceptible to these infections were shown to be biologically different in enterobacterial carriage on the vaginal mucosa than women resistant to the infection; the importance of the biological carriage was emphasized. Further data revealed that onset of bacteriuria was common following hysterectomy. Studies in rabbits indicated that hysterectomy has a substantial influence on vaginal colonization with *E. coli*. For the first time, therefore, a potential adverse effect from an otherwise uncomplicated hysterectomy procedure may be indicated.

AI-07826-06 F. A. Kapral (Ohio State University)

Overgrowth of staphylococci in the gut after broad spectrum antibiotic therapy is not uncommon; effects may range from mild diarrhea to severe enterocolitis. Results of this study on staphylococcal delta toxin in guinea pigs has shown that, unlike cholera toxin, no significant stimulation of adenyl cyclase activity occurs. The delta toxin did, however, cause a fluid and electrolyte loss in the gut, primarily from the mucosa. This is in contrast to the classical food poisoning staphylococcal enterotoxin, which has no such effect. Another part of this same project on staphylococci is on exfoliatin, a toxin of *S. aureus* causing a severe exfoliative dermatitis, especially in children, which has been called the "scalded skin" syndrome. Results of this study showed that significant skin loss could occur even after initiation of chemotherapy to destroy the staphylococci, because of toxin already produced by the organisms. Specific antitoxin therapy promptly halted this exfoliation in neonatal mice. Hyperimmune antiserum could therefore be an important adjunct to chemotherapy of this infection.

AI-05022-11 G. S. Bulmer (University of Oklahoma)

Cryptococcosis, a frequently fatal fungal disease of man, is caused by an encapsultated yeast that attacks the brain meninges. This pathogenic yeast is ubiquitous in occurrence; furthermore, the number of cases diagnosed appears to be increasing. One of man's potent defense mechanisms against this organism is myeloperoxidase (MPO), an enzyme found in leukocytes. Recent findings in this study indicate that fungal cells cultured in soil had a greater survival rate when exposed to human MPO than did fresh isolates. The soil apparently inhibited the MPO activity. Additionally, older yeast cells, obtained from within walled-off lung lesions, were more resistant to the action of MPO. Human MPO is lethal for several other species of fungal pathogens; for the MPO to be fungicidal, a balance must be maintained between concentration of the MPO and iodide and peroxide concentrations. It was also found that an anti-cryptococcal effect occurred to a much greater extent in saliva from normal volunteers than in saliva of patients undergoing immunosuppressive, steroid or antibiotic therapy.

AI-08715-02 D. Abram (University of Pittsburgh)

Bdellovibrio is a bacterial parasitic microorganism that parasitizes its host cell, the intestinal bacterium E. coli, and selectively attacks and destroys it. This natural parasite is of fundamental interest, although it cannot be predicted yet whether any therapeutic potential for this microorganism exists. Results of this study show that the envelope of the bacterial host cell is physically disrupted, causing functional cell damage and permeability changes. The initial damage to the bacterial host cell by the parasite appears to be to host cell permeability, without any preliminary detectable disruption of the host cell envelope. Following this, a lesion is formed which causes more extensive disruption of the host protoplast. Host cell enzymes are released and the host cell is emptied of its cytoplasmic contents, leaving behind empty "ghosts." The data also indicate that the host cell is killed early during the interaction, before the disruption of the outer wall membrane.

III. VIRAL DISEASES BRANCH

The approximate level of research support in Viral Diseases:

Type of Grant	Number	Amount
Research Grants 1/	254	$14,374,772
Training Grants	18	1,309,489
Fellowships	45	500,357

Summary

The role of viruses as a causal agent in many infectious diseases is well established but in others it is not so well known and continues to be a matter of concern and increasing importance in health problems today. The accumulation of information by our grant supported programs will contribute to improved ways of understanding, preventing and treating the variety of serious infections these viruses cause. Important and necessary basic information is being acquired that will lead to a reduction of the severity of illness and mortality.

The Viral Diseases Branch programs now include five special emphasis programs. These are: Chronic and Degenerative Diseases of Man, Antiviral Substances, Viral Hepatitis, Clinical Virology and Influenza. Important and necessary steps were made this past year that are contributing to our understanding of these diseases and hopefully may lead to eventual control of some viral diseases. For example, the identification of Hepatitis A and Hepatitis B viruses have improved vaccine possibilities and help to elucidate the long-term effects and pathogenesis of these diseases. Also, the progress being made in developing experimental systems for investigating the pathogenesis and etiology of chronic and degenerative diseases should clarify some of the long-term problems relating to these diseases. In another area, research is being focused on identification and characterization of effective antiviral substances and exploitation of already known antiviral substances and drugs. Research in clinical virology is being supported to provide an environment that will favor and enhance collaboration between investigators well-trained in concepts and methods of modern virology and clinical investigators with a substantial continuing responsibility for the diagnosis and treatment of patients with viral diseases. Progress is continuing in studies on influenza virus toward developing more acceptable vaccines as well as elucidating aspects of the biology of these viruses.

A. Viral Hepatitis (SEP)

Progress is being made in the understanding of the agents of viral hepatitis. The agents of hepatitis A and hepatitis B appear to be transmitted by a variety of methods. Investigations on these have resulted in improved or new methods for detection and study of these

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1/ Includes 26 career awards ($615,555).

diseases and in new information that hopefully will lead to control.

AI-10176-03 P. Kohler (University of Colorado)

Investigators on this project reported that prompt specific antibody treatment of infants born to mothers with hepatitis B virus infections prevented chronic neonatal infection. The physicians treated four infants whose mothers' hepatitis B virus infections were determined by the presence of hepatitis B surface antigen (HB_s Ag) in their blood. Two of the women had acute infections at or immediately before delivery; the other two had asymptomatic chronic infections. Their infants were treated with specific human antibody to the hepatitis antigen (anti-HB) within two to six days of birth. Regular testing up to the age of 16 months revealed no sign of hepatitis antigen in any of the infants. The results of a control group of seven mother-baby pairs were quite different. Six of seven untreated babies born to antigen-positive mothers were antigen-positive for 5 to 36 months. Since antibody treatment does not arrest established infections, the excellent protection of antibody prophylaxis in these infants indicates that they were not infected in the womb. These studies suggest that the reservoir of chronic hepatitis B virus infections or carriers could be reduced if women were tested at term and the infants of antigen-positive mothers given prophylactic antibody treatment.

B. Influenza (SEP)

This infectious disease continues to persist as a widespread epidemic threat in the United States. New knowledge of viral structures and genetic constitution has stimulated new approaches to more effective vaccine development against influenza.

AI-08831-06 R. G. Webster (St. Jude Children's Research Hospital)

The biological, biochemical and immunological evidence only provide circumstantial evidence that antigenic shift in human influenza viruses may occur by recombination. This project hypothesizes that more definitive evidence could be obtained by detecting recombination between different influenza viruses in nature leading to the appearance of a new pandemic strain in man. The approach to this problem is to isolate influenza viruses from animal and bird populations in advance of the occurrence of the next human pandemic strain; that is, to establish a bank of influenza viruses. After the appearance of the next human pandemic strain the virus can be compared with the viruses in the bank and thus determine if recombination plays an important role in the emergence of new human strains in nature. The wild animal and bird populations have largely been ignored as a source of new influenza viruses. In terms of evolution, bird populations of the world have lived in high density in their breeding colonies for much longer periods than terrestial animals. It is of interest that there are eight different subtypes of avian influenza viruses already isolated, six from domestic birds. It appears the logical place to start looking for influenza viruses in nature is in large bird colonies, particularly at the end of the nesting season - these studies are underway. The ecological approach to influenza viruses would establish the number of different subtypes that exist in nature and may offer definitive evidence of the origin of new strains. If there are a finite number of influenza A viruses, then in the future it may be possible to think in terms of control of the last remaining great plague of man.

AI-09304-06 E. D. Kilbourne (CUNY Mt. Sinai School of Medicine)

As with few other diseases the answers to influenza lie in a definitive understanding of the causes and nature of genetic (antigenic) variation of the influenza virus. These basic studies of the genetics of the virus are conducted with a view toward achieving insight into the nature of antigenic variation as well as the source of new variants in nature. The isolation of the viral antigens in pure form is a step in this direction and also provides yet another approach to artificial immunization. In these investigations intensive study of certain recombinants of identical serotype but with differing expression of neuraminidase and hemagglutinin functions, as well as ts mutants with specific deficiencies in these functions have provided a wedge for a specific dissection of viral structure and function. The use of recombinational selection techniques permit the segregation of other influenza virus polypeptides for discrete analysis of their composition, antigenicity, structure and function. Other studies are planned for approaching the problem of antigenic variation which will also help in obtaining better immunization materials. Definition and analysis of mutant complementation and recombination groups will be continued. As a specific practical problem investigations will continue on the nature of those genes associated with high yield in vaccine strains of virus.

AI-04360-11 G. K. Hirst (Public Health Research Institute)

In these investigations mixed infection of chick embryo fibroblasts with temperature sensitive mutants of WSN strain influenza A virus resulted in a number of recombinant-yielding cells far in excess of what was predicted in terms of adsorbed multiplicity or of the plaque-forming virus employed. These results reinforced the previously proposed conclusion that non-plaque-forming particles are the principal source of recombinants in mixed infection. The results strongly suggest that non-plaque-forming virus has the capacity to enter cells and generate some syntheses such as the formation of messenger RNA and viral structural proteins. They also suggest that two non-plaque-forming particles, each bearing a mutation on a different RNA piece, may give rise to wild-type influenza virus by means of complementation and recombination.

On the basis of complementation-recombination patterns a number of mutant strains have been classified into eight groups. Members of each group give high frequency complementation-recombination with each of the other groups. The physiological basis of the classification is being investigated.

The problem of genetic classification of influenza is proving to be more complicated than had been anticipated. The variability in mutant behaviour, the frequent occurrence of multiple mutations and especially the preliminary work on the nature of mutant defects in biochemical terms has strengthened this feeling. Defects in neuraminidase production and in the synthesis of M protein has been found to cut across line of genetic grouping. It is believed biochemical studies will assist in clarifying the meaning of recombination groups.

C. Antiviral Substances (SEP)

The support of research on antiviral substances is being focused on identification and characterization of effective antiviral agents and further exploitation of already known antiviral substances and agents.

AI-04059-12 G. G. Jackson (University of Illinois Medical Center)

A double-blind, placebo-controlled study was performed to observe the effect of a topical interferon inducer in the prevention of rhinovirus infection (colds) and illness in 30 young adults. The drug, a substituted propanediamine, was administered as nose drops for two days, beginning one day before challenge with rhinovirus Type 21. Interferon subsequently appeared in nasal washings in a high titer. Among antibody-free persons drug treatment, as compared to placebo, decreased infectivity of the virus, markedly reduced symptomatic illness, and decreased virus shedding if infection occurred. Among subjects with prechallenge antibody, drug treatment further increased resistance to infection, decreased virus recovery, and prevented symptoms. The data show a statistically significant drug-related and interferon-related protection against illness caused by rhinovirus Type 21, a common-cold virus.

In separate controlled studies, treatment of rhinovirus infected persons with aspirin decreased the symptoms of infection insignificantly, but significantly increased virus shedding. The investigators pointed out that for mild illness, the effect must be considered adverse.

AI-05629-12 T. C. Merigan (Stanford University)

A feline interferon production and assay system has been defined which allows measurement of the interferon produced by a single cell. The presence of trypsin or pretreatment of the cells with actinomycin D, an inhibitor of protein synthesis, eliminated the effect. The antiviral effect was specific, and cell-free supernatants from target cell monolayers which had been exposed to induced cells could be used to protect other target cell monolayers. The number of cells required to protect target cells was found to be inversely proportional to the amount of interferon produced in monolayer culture.

These results demonstrate that, under certain conditions, it is possible to measure the effect of the interferon produced by isolated single cells. There are immediate applications of this method to the study of interferon production in heterogeneous cell populations. Here, the ability to physically isolate the individual cells producing interferon from the nonproducers in the population would lead to an unambiguous assignment of the interferon inducing function to a particular species of cell. In addition, direct correlations can be made between antiviral effects in target cells and biochemical events occurring in the individual interferon-producing cells. The results will aid in resolving some of the problems related to possible application of interferon in the future.

K04-AI-36028-02 M. S. Finkelstein (New York University)

Investigations on this project show that plant lectins are effective antiviral agents in vitro. As others have reported, it was confirmed that lectins agglutinate a variety of enveloped viruses and cells infected with both enveloped and nonenveloped viruses. Lectin-produced inhibition of virus release was strongly suggested by the rapid development of antiviral effect against an enveloped (Sindbis) and a nonenveloped (polio) virus. Lectins were also shown to agglutinate cell-associated virus; this together with inhibition of release, seemed the most important antiviral mechanism contributing toward reduction of virus yields from lectin-treated cells. Lectin toxicity appeared minimal, and lectins at low concentration could protect monolayers, at least partially, against the development of cytopathic effect. It is hoped that future studies on these will be able to define more precisely the lectin relationship to replicating virus.

AI-09775-04 J. Fox (University of Washington, Seattle)

During outbreaks of influenza, people who are already ill are at the greatest risk of developing a severe flu infection with complications which can sometimes lead to death. Hospitalized patients are expecially vulnerable. A study now reveals that amantadine hydrochloride - an antiviral drug - can prevent hospital-acquired influenza infections.

Patients admitted over a 30-day period to the medical and neurology services at the Harborview Medical Center, Seattle, were included in the influenza study. Treatment and nontreatment groups were established solely on the basis of hospital record number. Amantadine hydrochloride - 100 milligrams orally every 12 hours - was prescribed upon admission for patients with odd number hospital records and was continued uninterrupted through the day of discharge.

None of the 50 patients who received amantadine had clinical symptoms of influenza and only two had subclinical symptoms. In contrast, of the 61 patients who did not receive amantidine, seven had clinical influenza and five had subclinical infection. These results indicate that amantadine prevented infection in the treated patients, since 12 of the 14 who developed infections were not receiving the drug. There were no major side effects from the drug. As a result of this study, the investigators recommend that, when influenza is present in the community, serious consideration should be given to the use of amantadine hydrochloride in hospitalized patients.

D. Clinical Virology (SEP)

This support program is to provide a research environment that will aid collaboration between investigators well-trained in concepts and methods of modern virology and clinical investigators with a continuing responsibility for the diagnosis and treatment of patients with viral diseases. It is hoped to provide a better understanding of the pathogenesis of major viral diseases in man to develop more effective methods of prevention, diagnosis and treatment.

AI-09785-04 A. H. Ross (Nassau County Medical Center, New York)

Zoster immune globulin is the first effective therapeutic agent for both prevention and attenuation of varicella (chickenpox) in exposed high-risk and moderate risk individuals who are immunologically normal. This is a specialized immune serum globulin, derived from the plasma of convalescent herpes zoster donors in otherwise normal health. The basis for its development is the higher and more consistent rise in varicella-zoster antibody after herpes zoster (shingles) than after varicella, since herpes zoster represents reactivation of latent varicella-zoster virus at a variable interval following the primary attack of chickenpox. Until a vaccine or a highly effective chemotherapeutic agent supplements or replaces zoster immune globulin, this will probably remain the first line of defense against varicella. Patient groups studied, in evaluating zoster immune globulin, included high-risk (immunosuppressed), moderate-risk (debilitated), and age-dependent risks (neonatal, adolescent, and adult) exposed in ambulatory or hospital settings.

AI-08731-05 J. C. Neiderman (Yale University)
AI-11611-02 D. Horstmann (Yale University)

Scientists supported on these projects report that excretion of Epstein-Barr (EB) virus is three times more common in renal transplant and other immunosuppressed patients than in untreated individuals. Although the clinical and epidemiological significance of this finding has not been determined, it supports the hypothesis that suppressive drugs reactivate latent EB virus infections. The fact that this reactivation occurs in the presence of humoral antibody indicates that a breakdown of cell-mediated immunity is involved.

It has been previously established that patients with infectious mononucleosis shed the EB virus for many months after the acute illness. This investigation was designed to see it this was also true of renal transplant and other immunosuppressed patients, since this information might shed light on natural defense mechanisms by which the viral carrier state is terminated. Immunosuppressive drugs are used when it is necessary to overcome the body's immune mechanisms, as in organ transplantation. These drugs are also used to treat autoimmune diseases in which the body, for still unknown reasons, begins to destroy its own cells or organs. Patients on these drugs become susceptible to many infections, particularly by the herpes group of viruses. EB virus is characterized as a herpes virus.

E. Chronic and Degenerative Diseases of Man (SEP)

Some of the major problems of research on the chronic and degenerative diseases have centered on recovering the causal agents, determining time of onset of the disease and defining host factors which influence the pathological evidence of disease. Research in this area has so far been largely in animals that serve as models for initiating new w approaches aimed at probing these problems in diseases of man.

AI-09628-05 F. W. Deinhardt (Rush-Presbyterian-St. Luke's Med. Ctr.)

The demonstration that some of the chronic diseases of the central nervous system of man are transmissible to nonhuman primates has strengthened the feeling that these and other chronic degenerative diseases of man may be of viral etiology. During this year the reliable serial passage of Kuru and Creutzfeldt-Jakob (CJD) disease was made to marmosets. The transmission of Kuru and CJD to marmosets has established a suitable model for the study of these diseases. Serial passage of these diseases in marmosets has resulted in shorter incubation periods (average 7-8 months) and provided needed biological material for future studies. This model may: (1) provide a better understanding of these diseases; (2) provide a system in which to identify the etiological agent of these diseases; (3) define an approach for studies related to diagnosis, treatment and prevention of these diseases, and; (4) stimulate additional interest and research in related chronic degenerative diseases.

AI-09022-06 A. Koestner (Ohio State University)

The overall objective of this project is to utilize the canine distemper complex as a model for studies of pathogenesis of a virus-induced demyelinating encephalomyelitis. In addition to subacute spongiform encephalitis, with which demyelinating distemper encephalitis has been compared, evidence has now accumulated that multiple sclerosis may also be a virus-induced demyelinating encephalitis. Most serological surveys have indicated a high measles antibody titer in multiple sclerosis patients as compared to control populations. Viral particles of the paramyxovirus type have been demonstrated in lesions in cells cultured from brains of multiple sclerosis patients. In the light of these developments the successful experimental production of demyelinating encephalitis in gnotobiotic pups with a special canine distemper virus isolate has gained markedly in importance as a model of virus-induced demyelination in man and should aid very much in future investigations on these diseases.

AI-09118-05 J. E. Craighead (University of Vermont)

There has been substantial evidence that heredity plays a role in the development of human diabetes. There has also been evidence that certain viruses may provoke onset of the disease, even though only a small fraction of infected individuals are affected. Since genetic factors influence susceptibility to viruses such a genetic influence on the response to diabetes-causing viruses may be one mechanism by which heredity affects the occurrence of diabetes. To examine this proposition, scientists untertook to study the effects of a diabetes-inducing virus in mice of varying genetic constitutions. The agent - encephalomyocarditis or EMC virus - resembles viruses of the type implicated in diabetes causation in man and produces a disease in mice which exhibits many features of human juvenile-type diabetes.

The nature of the hereditary factors influencing development of diabetes in EMC virus-infected mice remains unknown. Observed differences between strains might reflect something as general as inherited variations in metabolism or as specific as genetic differences in susceptibility of insulin-producing cells. Regardless of mechanism, however, the studies furnish a model for future study of viral induction of diabetes limited to genetically susceptible individuals.

F. Acquisition of New Knowledge

It is essential that there be continuous gathering of new knowledge on known disease producing viruses that continuously present a threat as well as detecting and characterizing new viral agents that may appear at times. For example, there are a variety of arboviruses capable of coming up with surprise epidemics (example - the recent spread of Venezuelan equine encephalitis from Central America north to Texas) and can be spread with ease by well established vectors. Also different approaches in investigations of viruses give information pertinent to better understanding of their pathogenesis, antigenic change or basic components, and often lead to more efficient and acceptable disease control possibilities.

AI-09706-05 H. Koprowski (Wistar Institute)

Evaluations were made of the efficacy of an antirabies vaccine produced in a human diploid cell culture (HDCS) when the vaccine is given after exposure to rabies virus. In an attempt to determine if the excellent protection conferred by the vaccine produced in baby hamster kidney (BHK) cells is the unique property of BHK cell-origin products or whether vaccine with similar activity could be prepared in other cells they studied vaccines produced on human diploid cell culture and on primary hamster kidney cells (PHKV). It was demonstrated that a single dose of antirabies vaccine prepared from virus propagated in HDCS culture can completely protect monkeys from a virulent challenge with street rabies virus administered several hours prior to vaccination.

Clinical studies of human diploid cell vaccine (HDCV) in human volunteers were evaluated by antibody responses in human volunteers in the United States, France and India following vaccination. A four dose vaccine schedule (0, 3, 7 and 14 days) appears to produce neutralizing antibody levels much higher than those observed after a full 14 - 21 day course of conventional vaccine now given on a daily schedule. This observation could provide a basis exposure rabies treatment of man.

AI-12203-02 F. Rapp (Hersey Medical Ctr., Uni. of Pennsylvania)

In studies of measles virus latency the investigators have demonstrated that genetic mutation can result in an alteration of the pathogenic properties of a virus. This experimental finding supports the hypothesis that a mutant or a virus variant can produce disease different from that normally associated with the wild-type virus (i.e., hydrocephalus).

The ability to predict the neurovirulent potential of a measles virus by its sensitivity to temperature confirms that these investigators possess a powerful probe for an understanding of the biological properties of measles virus. Furthermore, preliminary studies suggest that temperature sensitive mutants are useful in elucidating a mechanism for the persistence of a virus in cells without the virus killing the cell.

AI-11428-01 R. C. MacDonald (Northwestern University)

In studies on membranes and the action of viruses a method for introducing foreign antigens into live cells has been developed. This work shows that foreign antigens can be incorporated into the membranes of living cells by fusing lipid vesicles containing the antigen with the cell membranes. It was undertaken to provide direct evidence for the assertion that lipid vesicles of appropriate composition interact with cell membranes in much the same way as do viruses with lipid envelopes. In the studies, N-(dinitrophenyl)- phosphatidylethanolamine was incorporated in lecithin-lysolecithin-stearylamine bilayer vesicles and incubated with cells. The use of electron microscopy and ferritin-conjugated antibody to the dinitrophenyl antigen revealed that the antigen is very extensively incorporated into the cell membrane. Furthermore, antigen-containing vesicles were seen fusing with the cell membrane. It has also been shown that such cells are lysed in the presence of the antibody plus complement, which is further evidence that the antigen has been incorporated into the cell membrane. Cells so modified will permit studies on the immune response of the host to a cell that has a single defined antigenic component. Also, it will permit quantitative studies on the persistence of foreign antigens in cell membranes - of importance in viral pathogenesis.

IV. PARASITOLOGY AND MEDICAL ENTOMOLOGY BRANCH

The approximate level of support given Parasitology and Medical Entomology:

Type of Grant	Number	Amount
Research Grants [1]	166	$6,693,819
Training Grants	13	902,701
Fellowships	13	145,148

Summary

Since the last fiscal year (1974) report was prepared, two program evaluations have been conducted. The first involved the program "Biological Regulation of Vectors" and took place on June 17-18, 1974. The second involved the program "Immunity to Animal Parasites" and took place on October 24-25, 1974. As a result of the latter evaluation, the title of the program was changed to "Immunology of Parasitic Infections." The second evaluation was structured so as to be somewhat more "goal oriented" than the "Biological Regulation of Vectors" exercise.

A. Biological Regulation of Vectors (SEP)

For the past 30 years, control of pests and vectors of human disease has been based primarily on the use of synthetic organic compounds which had the "advantages" of long residual action and toxicity to a broad spectrum of target organisms. It has now been shown that because of these very characteristics many of these pesticides are more deleterious than beneficial, when all effects on man and his environment are considered. Furthermore, resistance to broad spectrum chemical pesticides has reduced their effectiveness in many vector control programs. For these reasons, the search for alternative methods of pest control has become imperative, and it is generally agreed that the best approaches will consist of integrated pest management programs which combine biological control, in the broadest sense, with judicious use of more specific chemicals and management of the physical environment. This type approach to vector control must be based on adequate information about the ecology of the target organism, the environment in which the control program is to be conducted, effects of control measures on non-target organisms in the environment, and the biology of the disease organisms being transmitted.

This program has as its goal the advancement of fundamental studies which might lead to effective methods of biological regulation of vectors.

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[1] Includes 9 career awards ($174,450).

Some of the conclusions and recommendations endorsed by the evaluators follow:

1. The identification of "Biological Regulation of Vectors" as a Special Emphasis Program (SEP) by NIAID has contributed significantly to the recognition of the need for research in this area, and to its stimulation, not only in the United States but internationally. Consequently the program should be continued and additional funds should be allocated to it to the extent possible.

2. The description of the Special Emphasis Program should be revised slightly to reflect more explicitly the importance of basic studies on biology and population dynamics as a part of the continuum of research and development required in this area before it can be determined if the promise of effective biological control can become a reality in economically feasible integrated pest management control.

3. Consideration should be given by NIAID to sponsorship of one or more workshops on topics which can be expected to be of increasing importance and concern as research in this area proceeds, such as "Evaluation of Hazards of Biological Control of Pests." This is currently under consideration by staff.

4. Each grantee working in this area should be encouraged to be very sensitive to potentially harmful environmental effects of any pathogen being used in experimental laboratory studies, to keep alert to evolving standards and regulations for safety requirements, and to incorporate in early phases of the research adequate preliminary studies to provide reasonable assurance of safety to man and non-target species as the experiments progress on the primary effects on target species. This is highly desirable in order to avoid expenditure of considerable time, effort and funds, only to find that an effective control procedure may be impractical or unacceptable because of its harmful environmental effects or direct toxicity to non-target species or man.

   Likewise each application for support of work in this area should be considered very carefully for adequate attention to concerns about potential harmful effects of proposed control activities, such as effects on existing flora-fauna, the introduction of vectors capable of transmitting other diseases, etc.

5. Consideration should be given to encouraging the development and maintenance by an appropriate government entity (perhaps the Federal Working Group on Pest Management) of a better data base on research activities in this area by

Federal and state laboratories/institutions and through grants and contracts.

6. The combined results of the projects in this program are exerting a major impact on the field of vector-borne diseases and pest management generally. Considering the short history and evolution of the program, the balance of work is judged to be quite good, reflecting considerable seminal activity over a broad spectrum of the field. However, certain lacunae exist in which much more work is urgently needed, e.g., life table studies.

7. NIAID should continue to concentrate its limited resources for work in this area on the more fundamental, exploratory studies to identify potential bio-control alternatives and evaluate these through pilot field tests of both effectiveness and safety. In this context, it was noted that there appears to be no explicit assignment of responsibility to any agency for taking the more promising control measures thus identified through the necessary large-scale field trials, safety testing, and mass-production technology development. As work in this area proceeds, this could be a significant deterrent to the exploitation of promising control measures. Therefore, consideration should be given to this important aspect of national policy.

Specific examples of research progress during the current fiscal year follow:

AI-05882-13 H. C. Whisler (University of Washington, Seattle)

Of the various pathogens for the control of mosquitoes under study in this program, fungi of the genus Coelomomyces are probably the most promising candidates.

Drs. Howard C. Whisler and Stephen L. Zebold, from the University of Washington, report that an intermediate host is necessary in the life cycle of the infective fungus, Coelomomyces psorophorae. This knowledge has enabled the investigators to infect the larvae of the mosquito Culiseta inornata under relatively simple laboratory conditions. They found that the spores (or seeds) of the Coelomomyces require two different hosts: first, a copepod (a minute marine animal) called Cyclops vernalis, and second, the mosquito larvae. Spores from the mosquito larvae then infect more copepods. Thus the hosts must alternate for the spores to grow. This alternation of hosts, called heteroecism, is common in some other fungi, but has not been noted previously in the large class of algae-like fungi called *Phycomycetes*, to which the *Coelomomyces* belong.

Drs. Whisler and Zebold are now investigating other species of *Coelomomyces*. They are also seeking ways of using this information for the biological control of large populations of mosquitoes, including those that carry the organisms causing malaria and yellow fever. Although the mosquito used in this study, *Culiseta inornata*, has not been identified as a disease carrier, its close relative *Culiseta melanura* does carry the Eastern equine encephalitis virus, which causes sometimes fatal encephalitis in humans, as well as in horses and wild birds.

Over the years many different strains of *Coelomomyces* have been recognized as natural enemies of different species of mosquitoes. *Coelomomyces* seems to be quite specific for mosquitoes and, therefore, without harmful effects on other insects or on man. When found naturally, the fungus maintains itself in the mosquito environment year after year providing a continuous control on mosquito population growth. However, past attempts to control mosquitoes by introducing *Coelomomyces* into the environment, for instance in the South Seas areas where malaria is a major problem, have been unsuccessful.

Now that the necessity for an intermediate host has been recognized, the possibilities for biological control of mosquitoes are considerably improved. Drs. Whisler and Zebold hope that introduction of the copepod as well as the *Coelomomyces* to an experimental situation will enable the fungus to follow its normal life cycle and to infect mosquitoes as it does under natural conditions. This method of biological control may thus diminish populations of disease-carrying mosquitoes without the use of chemical insecticides.

AI-10274-03 A. Spielman (Harvard School of Public Health)

This grantee observed that the Giant Tree Frog (*Hyla septentrionalis*) is important in regulating the abundance of the Southern House Mosquito (*Culex pipiens quinquefasciatus*) on Grand Bahama Island. This avidly man-biting pan-tropical mosquito is a notorious vector of human disease (urban filariasis and St. Louis Encephalitis). Both the frog and mosquito deposit eggs in relatively permanent collections of water around human habitations. It was found that tadpoles of these frogs are effective predators on larval mosquitoes, eliminating them from more than half of the otherwise suitable available breeding sites in the study area. The role of anuran tadpoles in regulating the abundance of mosquitoes has been ignored in recent years and these findings suggest that the subject be systematically explored.

Another appropriate finding is the discovery of a new species of peri-domestic mosquito (*Aedes bahamensis*) on Grand Bahama Island. This abundant autogenous mosquito rarely feeds on man in nature and does not support multiplication of 4 *A. aegypti*-borne arboviruses (including dengue). It has been found that *A. aegypti*, the vector of dengue and yellow fever, is present on Grand Bahama Island but its distribution is limited to two towns there. *A. bahamensis* is generally distributed in inhabited areas throughout the island but is not present in those towns in which *A. aegypti* is found. This differential distribution has been stable over a nine year period which suggests that populations of these species may be mutually exclusive.

TI AI-00046-14 E. Michelson (Harvard School of Public Health)

A North American snail, *Lymnaea emarginata* has been observed to control laboratory populations of *Biomphalaria glabrata*, a schistosome snail host, by destroying the eggs of the latter. Previous exposure to *B. glabrata* egg masses accelerated predation by *L. emarginata*. This phenomenon was considered to be due to sensitization rather than conditioning, since repeated exposure could not be correlated with an increase in the efficiency of predation. The predator was able to distinguish between the egg masses of *B. glabrata* and those of *Helisoma caribaeum*, and destroyed primarily those of the former; however, neither the egg masses nor their products appeared to attract the predator. While these findings are preliminary, the use of this snail as a possible agent for the biological regulation of a schistosome vector warrants further exploration.

B. Immunology of Parasitic Infections (formerly "Immunity to Animal Parasites")

The complexity of structure and function of parasites has made the study of immunology of these infectious agents exceptionally challenging and rewarding. Exciting opportunities for the elucidation of mechanisms and manifestations of immunological responses to parasites now exist as the result of the impressive developments in immunology in recent years.

Major ultimate goals of studies on immunology of parasitic infections are the development of effective vaccines for the prevention of parasitic diseases (such as malaria, schistosomiasis and filariasis), the intervention in the host response to prevent or ameliorate disease processes which are immunologically mediated, and the development or improvement of immunodiagnostic procedures for parasitic infections, especially as they relate to the immune status of the host.

A related goal of these studies is to contribute to an understanding and solution of basic and clinical problems associated with other disease entities, especially immunological disorders and hypersensitivity states. A number of parasitic infections are excellent models for such studies as: (1) the mechanisms of intracellular immunity; (2) the enhancement or suppression of concurrent infections or tumor development; (3) immunopathological mechanisms; (4) development of disease processes in immunosuppressed or immunostimulated hosts; (5) the biochemical and genetic mechanisms for the development of pathogen variants with different immunological characteristics; (6) the genetic basis for variations in host response; and (7) the role of IgE and other cytotropic antibodies in hypersensitivity.

Projects directed toward the accomplishment of the above stated goals involve a broad spectrum of studies, including: (1) the identification and characterization of protective and immunopathogenic antigens from components of parasites and their by-products; (2) the nature and mechanisms of host responses to the infection, with particular emphasis on both humoral and cellular immune factors and their interactions; (3) the effects and mechanisms of these effects of the immunological response on the parasite inciting the reaction, on concurrent infections with other organisms, and on the host itself.

Currently this Institute has an investment of 40 awards at $1.7 million in the Special Emphasis Program "Immunology of Parasitic Infections."

On October 24-25, 1974, this program was evaluated by a group of experts who were impressed with the vigor and breadth of the research in progress in this program. They attributed much of its strength to the cooperative efforts of parasitologists and immunologists in applying recent developments in immunology to the complex host-parasite relationships characteristic of protozoan and helminth infections.

Some of the conclusions and recommendations of the evaluators follow:

1. It was agreed that many of these infections--by virtue of the size, structure and physiology of the parasites--constitute excellent models for the study of immunological principles applicable to a broad spectrum of infectious, allergic, neoplastic and metabolic diseases. Therefore, it was suggested that NIAID officials consider sponsoring a workshop which would bring together a limited number of

key workers in these disciplines to explore ways of using more effectively models which have been established and to identify problems for which new or better models are needed and which might be developed from among the great diversity of parasitic infections.

2. Recognizing the extent and importance of parasitic diseases throughout the world, the responsibility of NIAID to support research in this area--not only for improvement and protection of the nation's health directly, but also under its Legislative mandate to support research in international health to assure achievement of this mission; the progress being made toward realization of the program's goals; and the role of the program in stimulating research in this area, the panel recommended that this Special Emphasis Program be continued with a change in title as indicated.

3. Inasmuch as one of the greatest problems impeding progress in this area is the difficulty of securing adequate quantities of materials with which to work, it was suggested that consideration might be given to encouraging innovative studies on the possible modification of certain free-living organisms in such a way as to make them parasitic and thus to provide better models for some studies. Such an approach need not be considered as lacking in promise in view of the very close relationship of many free-living and parasitic organisms.

4. It was concluded that since many of the principles operative in the immunological responses to parasites are basically similar regardless of the type of parasite, it is desirable to continue supporting research in a broad range of organisms and hosts. At the same time, recognizing the realities of budget limitations and the greater urgency in developing effective control measures for major parasitic diseases, the panel suggested that the Institute staff consider developing plans for both careful monitoring of the advances in world-wide research on certain of these diseases (e.g., malaria, filariasis and schistosomiasis) and more effective marshalling of resources to exploit major developments which could lead to achieving program goals when the opportunities become available.

5. With respect to the monitoring of research progress on immunology of a given disease (e.g., schistosomiasis) it was suggested that the Institute might explore development of a mechanism for cooperative effort with other national and international agencies providing significant support for research on that disease to effect interchange of appropriate information, thus permitting better cognizance of current developments. After a reasonable trial period, such a program could be terminated or expanded to cover other diseases, based on the experience with the model system.

6. The panel emphasized the importance of appropriate studies on humans with diseases such as schistosomiasis and recommended that every effort be made to assure availability of funds for travel by investigators to endemic areas of these diseases. Furthermore, it was suggested that NIAID staff explore all possible avenues of cooperative efforts to promote work on these diseases with such diverse organizations as the Fogarty International Center of NIH, AID, DOD, the Rockefeller Foundation, the Wellcome Foundation and WHO.

Specific examples of research progress during the current fiscal year follow:

AI-12317-01 M. Boyer (Harvard)

Experiments in rhesus monkeys indicate that the parasite *Schistosoma mansoni* evades destruction by the host immune response by incorporating into its surface antigenic determinants similar to those of the infected animal. Schistosomes grown in other (donor) mammals and transferred into the portal systems of monkeys are rapidly destroyed in a "second set" type reaction if the recipient has been immunized previously with donor tissue. The development in this laboratory of a surgical procedure for transfer of adult schistosomes to the portal system of mice allows study of the "host antigen" phenomenon in murine schistosomiasis. Parasites from genetically defined, inbred strains of mice have been transplanted to the portal system of other mouse strains in an effort to understand the genetic and immunologic factors responsible for schistosome acceptance or rejection.

AI-4717-13 J. Remington (Palo Alto Medical Research Foundation)

Human macrophages derived from *in vitro* culture of peripheral blood monocytes were studied under a variety of conditions to determine their microbicidal capacity for the obligate intracellular protozoan, *Toxoplasma gondii*. The effect of macrophages on intracellular *Toxoplasma* was evaluated morphologically by light and phase microscopy and by autoradiography. When macrophages from dye test (DT)-negative or DT-positive individuals were infected with *Toxoplasma* in the presence of normal human serum, the organisms were able to multiply intracellularly with resultant destruction of the monolayer. Once organisms were intracellular, the presence of antibody-containing serum in the medium did not alter this inability of the macrophages to kill *Toxoplasma*. However, when *Toxoplasma* were incubated in the presence of heat-inactivated DT-positive serum just before infection of the monolayers, the intracellular organisms were inhibited or killed by normal macrophages. Attempts were made to activate macrophages *in vitro* to kill *Toxoplasma*. Macrophages incubated in the presence of sensitized lymphocytes and Streptokinase-Streptodornase (SK-SD) or *Toxoplasma* lysate antigen (TLA) were found to kill *Toxoplasma* when compared to macrophages incubated in the presence of lymphocytes from DT-negative individuals and TLA or lymphocytes alone. Thus, *in vitro* induction of resistance (both specifically and nonspecifically) in humans macrophages was accomplished by culturing these cells in the presence of specifically sensitized lymphocytes and antigen. These results suggest that, as in the mouse model, activated human macrophages have the ability to inhibit or kill intracellular *Toxoplasma* and that these cells may be important as effector cells in cell-mediated immunity (CMI) to toxoplasmosis in man.

AI-8163-07 K. Warren (Case Western Reserve University)

Cholera toxin (CT) injected intravenously into mice resulted in a raise in cyclic AMP levels in splenic white cells. This was associated with and followed by a marked and relatively prolonged decrease in both the spleen cells and circulating lymphocytes. The effect of CT on a series of cell-mediated immunologic reactions related to the pathogenesis of schistosomiasis was then tested. CT profoundly suppressed dermal footpad swelling to soluble schistosome egg antigens, granuloma formation around schistosome eggs injected into the pulmonary microvasculature of unsensitized and sensitized mice, production of macrophage migration inhibition factor by intact granulomas (obtained from the livers of infected mice) maintained *in vitro* in tissue culture, and it ameliorated portal hypertension and esophageal varices in hepatosplenic schistosomiasis. In addition CT significantly prolonged allograft survival in mice even though it was first administered 6 days after grafting. Finally, CT completely eliminated foreign body granuloma formation around plastic beads (used as controls for the egg granulomas) which is a nonimmunologic reaction due to activation of chemical mediators of inflammation.

C. Acquisition of New Knowledge

Examples of research conducted during the fiscal year follow:

AI-4717-13 J. Remington (Palo Alto Medical Research Foundation)

The effect of clindamycin on survival of mice during acute infection with the PH and C56 strains of *Toxoplasma* and the ability of this drug to prevent congenital transmission during the acute stage of the infection in the mother and to eradicate the parasite from tissue of mice chronically infected with the C56 strain, were evaluated. The drug effectively prevented death due to the acute infection and, in the experimental model employed, eradicated the organism at least from the liver, spleen, and brain of approximately 30 to 50% of the acutely infected animals which survived. Clindamycin also effectively prevented congenital transmission during the acute infection in the mother. During short-term treatment (7 days), persistent parasitemia in the chronic infection was effectively diminished or eradicated. More prolonged treatment resulted in a significant clearing of the organisms from the spleens and livers, but not from the brains, of chronically infected mice.

AI-11616-02 P. Morera (University of Costa Rica)

This investigator is continuing his pioneering exploration of the ecology of a new parasitic agent, *Angiostrongylus costaricensis*, a helminth which causes disease in man. The parasite has been isolated from 120 cases in Costa Rica, and has also been discovered in humans in Mexico, Guatemala, El Salvador, Honduras, Venezuela, and possibly Brazil. The definitive host is the rodent, and the intermediate host a mollusc. In the human the eggs of this worm are trapped in the ileocecal area where they produce granulomatous lesions with eosinophilic infiltration. The worm normally resides in the mesenteric arteries of various rats.

V. BIOCHEMISTRY AND PHYSIOLOGY BRANCH

The approximate level of support given Biochemistry and Physiology:

Type of Grant	Number	Amount
Research Grants 1/	167	$8,189,144
Training Grants	3	94,521
Fellowships	38	416,300

Summary

Early in July, 1974, the basic virology grants component of the branch was transferred to the Viral Diseases Branch. The Biochemistry and Physiology Branch now contains basic and fundamental research in the areas of bacteriology and mycology. These research efforts support the disease-related activities of certain of the other branches, principally Bacterial and Mycotic Diseases. The branch receives extremely divergent, but relevant, research grants from areas ranging from toxicology to genetics and molecular biology. Material from approximately 25 study sections is involved.

A. Mechanisms of Resistance to Antimicrobial Agents (SEP)

During the past ten years, the importance of health problems of microbial resistance to therapeutic agents has become increasingly apparent. The relevance of this phenomenon to other interests of this Institute such as hospital-acquired infections of staphylococcal origin and investigations of the gonococcus is obvious. At this point there is substantial clinical and epidemiological evidence that development of this resistance, and especially plasmid (extra-chromosomal) mediated drug resistance, represents a major problem in medical care. Since most of the projects dealing with the development of drug resistance are of a basic nature and have been concerned with defining the molecular and biochemical basis for resistance, the Special Emphasis Program in this area has been transferred from the Bacterial and Mycotic Diseases Branch to the Biochemistry and Physiology Branch.

The principal goal of this Special Emphasis Program is to elucidate fundamental biological mechanisms involved in the development of drug resistance in microorganisms and to increase our basic understanding of this phenomenon. Specific goals of this Special Emphasis Program involve investigation of the origin, development, evoluation, expression and mechanism of drug resistance in a variety of specific microorganisms. Examples of microorganisms of particular interest are (but not limited to): Enterobacteriaceae, Pseudomonas, Neisseria,

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1/ Includes 8 career awards ($189,433).

staphylococci, streptococci, mycobacteria, mycoplasmas, and pathogenic fungi.

Currently this Institute has an investment of 40 awards at $1.8 million in the Special Emphasis Program--"Mechanisms of Resistance to Antimicrobial Agents" and 56 awards at $2.4 million in related bacteriophage research (bacteriophage can serve as a "vector" of recombinant DNA).

On February 6-7, 1975, this program was evaluated by a group of international composition. The evaluation was "goal-oriented." The panel members characterized the grantees as a surprisingly goal-oriented group, even though most of them are doing research of a basic nature. Contributions of this small number of grantees to the general area of biomedical science have been many. Some examples of these follow:

1. Perhaps the most outstanding of these contributions has been the technique of genetic engineering. While this technique is a monumental one, it is not devoid of potential hazards. One of our grantees is now able to transfer a gene or genes from a given species to a totally unrelated one, thus altering the genetic structure of the latter.

2. Work on plasmid-determined enterotoxins may offer a method of dealing with diarrheal diseases of bacterial origin.

3. The mechanisms of sulfonamide resistance in pneumococci have been elucidated at the molecular level.

4. The demonstration that bacterial mutation can increase the number of resistance factor copies; an explanation of the high resistance of certain strains to the antibiotic, ampicillin. This work could lead to the identification of mutants which would specifically inhibit resistance factor replication.

5. Incompatibility typing of plasmids can be used to identify the origin and evolution of plasmids and may be important for epidemiological studies of resistant bacteria in a hospital.

6. Another contribution leads to the possibility of cloning bacteria with a high enterotoxin or antibiotic production. This would be of great importance to pharmaceutical houses.

7. Mapping of resistance determinants in _Salmonella_, utilizing resistance factors. The structure of _Salmonella_ antigens is highly important for diagnostic work; aberrations in these antigens are often attributable to plasmids.

8. The entire molecular basis of bacterial resistance to aminoglycoside antibiotics (gentamycin and neomycin) has been determined by another grantee. This could culminate in a "tailoring" of antibiotics.

9. The mechanism of evolution of plasmids has been explored and may lead to future control of the spread of plasmids.

10. Rapid accurate enzymatic assays for antibiotics in patient sera have been produced.

11. An ultimate contribution may be the use of plasmids as the models of choice for the study of replication in general, including that of bacteria and viruses.

Specific examples of research progress during the current fiscal year follow:

AI-08190-07 J. K. McClatchy (National Jewish Hosp. & Research Ctr.)

The primary goal of this work is to elucidate the modes of action of several of the most widely used antimycobacterial drugs, including isoniazid, rifampin, ethambutol and pyrazinamide. However, the mechanisms of resistance, both natural and acquired, to these agents are also of interest.

Studies of acquired isoniazid (INH) resistance in Mycobacterium tuberculosis indicate that the drug is not taken up by resistant bacteria. Strain BCG rapidly incorporates ^{14}C-INH and is saturated within 2-3 hours after addition of 1 mcg/ml to a growing culture. An INH-resistant mutant of BCG (BCG-INH^R) does not take up the drug. In later experiments, sonic extracts of strain BCG and BCG-INH^R were prepared, injected into rabbits, and antisera produced. Using the antisera and antigens prepared from the two organisms in immunodiffusion tests, it was found that BCG-INH^R lacks a prominent precipitin band found in the wild-type organism. Thus, it appears that the INH-sensitive parent strain possesses a component that is diminished or absent in the INH-resistant mutant. Whether such a component is related to INH up-take and/or INH susceptibility has not been established.

Recently, resistance to pyrazinamide (PZA) in M. tuberculosis has been studied. It has been found that susceptible organisms take up pyrazinamide and convert to pyrazinoic acid (POA) in an acidic (pH 5.5) environment. The latter compound appears to be responsible for the biological activity of the drug since PZA-resistant bacteria do not convert PZA to POA. Pyrazinoic acid added directly to the culture medium has no effect on growth.

Rifampin (RMP) is an inhibitor of RNA synthesis in several strains of bacteria, including mycobacteria. Most strains of tubercle bacilli are inhibited by 0.01 mcg RMP/ml. Sonic extracts were prepared from strain BCG, an RMP-resistant mutant of BCG, and several atypical mycobacterial strains (*M. kansasii*, *M. intracellulare*, *M. avium*) and the effects of RMP on RNA-polymerase activities in the various strains determined. RNA polymerase activity in BCG was inhibited by 90% by 0.001 mcg RMP/ml while polymerase activity in the RMP-resistant strain was unimpaired by even 200 mcg RMP/ml. Activity was completely inhibited in sonic extracts of the atypical mycobacteria by concentrations of 0.05 mcg/ml. However, all of the atypical strains grew normally in the presence of 50 mcg RMP/ml in the culture medium. These findings suggest that strains of atypical mycobacteria are naturally rifampin resistant because the drug does not readily penetrate the cell and an intracellular concentration sufficient to inhibit RNA polymerase activitity is not achieved.

AI-10076-08 J. Davies (University of Wisconsin, Madison)

The initial goal of this research program was to identify and characterize the mechanisms by which bacteria and simple eukaryotic organisms develop resistance to a variety of antibiotics. Both clinical resistance and laboratory-induced resistance have been emphasized. The former has application to the development of new drugs and new approaches to antibiotic therapy that are designed to avoid the development of antibiotic resistance. The latter aspect was designed with the notion that mutations to antibiotic resistance can be applied in studies of the genetics and biochemistry of cell functions such as protein synthesis; this gives important information on the mechanisms of action of antibiotics and on the nature of their target sites.

Studies of antibiotic resistance in clinical bacterial isolates have led to the isolation and characterization of those R-factor and plasmid-coded enzymes involved in resistance to the aminoglycoside antibiotics. A variety of clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Providencia stuartii*, *Staphylococcus aureus*, *Serratia marcescens* and other strains have been obtained from various sources and analyzed for plasmid determined enzymes. To date *nine* enzymes have been identified and shown by mutation to be associated with resistance to antibiotics such as streptomycin, kanamycin, gentamicin, tobramycin, neomycin, butyrosin, etc. The enzymes have been identified by substrate range and inhibition studies and in most cases the product of enzymatic modification of the antibiotic has been recognized chemically.

AI-9079-06 W. K. Maas (New York University)

Incompatibility has been a major obstacle for the analysis of the genetic constitution of plasmids, including R factors. These studies have led to the recognition of incompatibility as a very specific and stable property of plasmids. Plasmids which are incompatible with each other are usually (but not always!) closely related on the basis of DNA homology. Because of this, incompatibility has become an important criterion in the classification of plasmids, especially R factors. Plasmids which are incompatible with each other (and presumably closely related) are said to belong to the same incompatibility group. So far about 25 incompatibility groups have been established among transmissible plasmids of enteric bacteria.

Because of its specificity, stability and ease of determination, incompatibility has become a valuable tool for epidemiological studies. It permits one to trace the distribution of a given R factor among different genera of enteric organisms and to follow the spread of an R factor into different geographical areas and human populations.

B. Recombinant DNA and Genetic Engineering

Since 1967, this Institute has supported the work of Dr. Stanley Cohen (AI-08619-08) currently located at Stanford University Medical School. Dr. Cohen originated the technique of gene transplantation by taking recombinant DNA from a South African toad and inserting it into a bacterial plasmid which is able to replicate within *Escherichia coli*. This research of Dr. Cohen is *directly responsible* for the recent series of meetings in which biologists have expressed a serious concern that some of these genetically engineered recombinant DNA arrangements could prove biologically hazardous.

The international meeting at Asilomar in late February, 1975, was held for the express purpose of determining the risk involved in the genetic manipulation of recombinant DNA. At this meeting the Institute was represented by two members of the NIAID Intramural Program, Drs. Andrew Lewis and Malcolm Martin. In March, 1975, a second important meeting was held at Squaw Valley, California, at which the Institute was represented by Dr. Irving P. Delappe. Two leaders in the field who are grantees of this Institute, Drs. Stanley Cohen and Donald Helinski, organized this meeting and NIAID contributed $4,000 to its support. While the subject of this meeting was research on bacterial plasmids, an entire evening was devoted to a discussion of possible biohazards involved in genetic manipulation.

The area of genetic manipulation of recombinant DNA is a direct consequence of the research this Institute has supported for the past 8 years.

This Institute currently has no representative on the NIH "Recombinant DNA Molecule Program Advisory Committee" which met, following the Asilomar meeting on February 28, 1975, in San Francisco to formulate safeguards dealing with this problem. In fact, it was appreciably after this meeting that we were accorded the privilege of receiving a copy of the minutes of the San Francisco meeting. Several of the grantees of NIAID are members of this committee, but not a single committee member is from the Institute which sponsors their research. We believe NIAID staff could make substantial contributions to the work of this committee and they need to be informed about all matters on which the committee will advise.

In addition, the NIAID Extramural Program believes that the Institute should consider a program in the area of recombinant DNA. Staff is prepared to formulate goals and content for such a program.

C. Acquisition of New Knowledge

Examples of research conducted during the fiscal year follow:

AI-05540-10 S. Bartnicki-Garcia (Uni. of California, Riverside)

For the first time, synthesis of yeast cell wall fragments in a test tube has been achieved. This accomplishment adds much to our understanding of cell wall manufacture and brings closer the possibility of assembling an entire wall in the absence of intact cells.

This study was undertaken with cell membrane fractions of the yeast form of the fungus *Mucor rouxii*. The yeast's wall-manufacturing enzyme, chitin synthetase, is associated with the membranes, and the scientists hypothesized that the enzyme would be liberated by exposure of this fraction to the basic substance of which *M. rouxii* cell walls are composed--in other words, the enzyme's substrate.

The investigators' hypothesis proved correct. Addition of the substrate, along with an "activator," at 0 C., caused about 15-20% of the enzyme activity to become soluble. When the soluble enzyme was subsequently brought to room temperature, fibrous material precipitated out of solution. Chemical and electron microscopic examination of this product revealed that it--like *M. rouxii* wall--was composed of small filaments (microfibrils) of the polysaccharide chitin. Thus, for the first time, assembly *in vitro* of normally constructed microfibrillar wall had been demonstrated.

In addition to demonstrating that cell wall microfibrils can be formed in the absence of living cells, these results support the belief that formation of wall microfibrils takes place at the cell surface rather than within the cell.

AI-03242-13 C. C. Brinton, Jr. (University of Pittsburgh)

For the past 13 years this grantee has pursued studies on the genetics, structure and function of bacterial pili utilizing *Escherichia coli* for the research. Recently Dr. Brinton has had a chance to apply the pili expertise, acquired under the sponsorship of this Institute, to the development of a serum test together with a vaccine for gonorrhea utilizing purified gonococcal pili.

His NIAID grant, together with a Research Career Development Award from this Institute, enabled him to develop the general technology of bacterial pili and to establish and maintain a laboratory devoted to pilus research. Had there been no previous characterization of bacterial pili, the nature of the virulence factor in gonorrhea might still be obscure.

Although this Institute did not support Dr. Brinton's work on the serum test or the vaccine, we may take credit for a major public health advance which was derivative from our long-term support of fundamental work on bacterial pili.

K04-AI-33032-04 P. F. Sparling (Univ. of North Carolina, Chapel Hill)

Dr. Sparling has continued his studies of an unusual *E. coli* mutant which is dependent on erythromycin for its growth. The locus for dependence on erythromycin is near minute 25 on the *E. coli* chromosome. A second locus for resistance to erythromycin is clustered with other ribosomal genes near minute 64. The originally isolated dependent mutant was found to be a double mutant carrying both the mutations for erythromycin resistance and erythromycin dependence. Dr. Sparling was unable to find a structural basis for erythromycin dependence but was able to demonstrate alteration of binding of labeled erythromycin to ribosomes from the dependent mutant, confirming that the mutation effects the 50S ribosome.

Mutations which affect the structure of bacterial ribosomes are of interest because they provide a convenient tool for studying the relationship between ribosomal structure and function.

Further research by Dr. Sparling disclosed two loci for resistance to the aminoglycoside antibiotic kasugamycin.

AI-7885-06 F. Jacob (Pasteur Institute)

This grantee, a Nobel Prize winner, has found that the development of teratocarcinoma does not impair immunization of mice against *Listeria monocytogenes*. Endotoxin injection a short time before tumor cell inoculation allows the growth of teratocarcinoma in non-syngenic mice despite immune stimulation. In contrast with this absence of impaired systematic immunity, teratocarcinoma cells were found to repulse macrophages *in vitro*. This effect on macrophages was also found with three other malignant cells and with trophoblast cells. *In vivo*, teratocarcinoma cells were found to impair local inflammation. These cells and other malignant cells are able to produce a compound(s) of molecular weight between 10^3 and 10^4, which prevents inflammatory reaction. These results suggest that mouse teratocarcinomas and other tumors by-pass the host immunological system of surveillance by at least two mechanisms: a direct toxic effect on macrophages and the release of an inhibitor of inflammation.

AI-3772-14 P. Murphy (Johns Hopkins University)

The overall objective of this research, initially pursued under the late Dr. William Barry Wood and currently his successor, Dr. Patrick Murphy, is a better understanding of the mechanisms involved in the pathogenesis of fever.

Rabbit leukocytes from peritoneal exudates and from blood have been stimulated to form leukocyte pyrogen in the presence of radiolabeled amino acids. The stimuli used were endotoxin, phagocytosis, and tuberculin. The crude leukocyte pyrogen samples were purified; pyrogen from exudate cells was rendered homogeneous; pyrogen from blood cells was still contaminated with other proteins. All the purified pyrogens were radioactive; and for all it was shown that radioactivity and pyrogenic activity coincided on electrophoresis at pH 3.5 and pH 9 in acrylamide and on isoelectric focusing in acrylamide. Furthermore, pyrogens obtained from exudate cells stimulated in different ways, or from blood cells and exudate cells stimulated with endotoxin, appeared to be identical. These results suggest that leukocyte pyrogen was synthesized *de novo* from amino acid precursors and that leukocytes made the same pyrogen whatever the stimulus used to activate them.

IDB

GMB

COLLABORATIVE

RRB

TIB

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Annual Report for FY 1975

Report of the Associate Director for Collaborative Research

The organization and staffing of Collaborative Research remained relatively stable and unchanged in FY 1975, reflecting the end-of-year personnel ceiling of 36 and very few departures. Dr. Paul Pedersen, USPHS Medical Director, joined the Transplantation and Immunology Branch staff in June 1974 in replacement for a departing staff associate. Dr. Pedersen's preceding assignments had been in the Bureau of Medical Services, the Bureau of Community Health Services and the Division of Foreign Quarantine. However, the inability to add new staff remains as the single most limiting factor in the continued development of existing programs as well as for the initiation of new work.

Collaborative Research programs continue to receive outside scientific advice from a variety of sources. The Infectious Disease Branch and Transplantation and Immunology Branch are counciled by formally constituted committees, while the Geographic Medicine Branch receives scientific advice from seven panels corresponding to the categorical U.S.-Japan Program components. The Research Resources Branch now operates with advisory inputs from workshops, ad hoc sources, plus a small number of consultants. Primary review of the International Centers for Medical Research 5 year renewal grant applications was accomplished in FY 1975 by a DRG-convened Special Study Section followed by enthusiastic approval by the National Advisory Allergy and Infectious Diseases Council.

The annual Collaborative Research program scientific review and evaluation for NIAID senior staff and invited participants was conducted in November 1974. An attempt was made to utilize the FY 1974 Collaborative Research Annual Report,with an appropriate supplement as the basic review document. While these reviews are generally regarded to be of high value to both Collaborative Research staff, Research Contract Approval Group (RCAG) members and the Institute Directorate the review and evaluation would be more appropriate earlier in the fiscal year or preferably at the end of the preceding fiscal year. This would facilitate earlier and hence more timely planning, make better use of the annual report and reduce staff time required in the preparation of supplementary documentation.

In contrast to a full review of Collaborative Research programs made by the NIAID Advisory Council in FY 1974, the FY 1975 Collaborative Research presentation to the Council was limited to a brief organizational/operational overview by the Associate Director for Collaborative Research, Dr. Howard A. Minners, and by presentation of four selected scientific topics by Collaborative Research staff: Dr. John Nutter - Allergens and Immunologic reagents; Dr. James C. Hill - Polysaccharide Vaccines; Dr. Franklin Tyeryar - Hepatitis Vaccine and Dr. Donald E. Kayhoe - Evaluation of Antilymphocyte Globulin.

Fiscal planning and program implementation in FY 1975 was complicated by a "Rescission" order which would have restricted Collaborative Research obligations to a level of $11,686,000 (gross) for contracts and agreements instead of the originally budgeted level of $12,888,000 (gross). "Rescission" was not withdrawn until March 15, 1975. As of the preparation of this report the net available for Collaborative Research contracts and agreements appears to be $12,100,000 which is about the same level of obligations as in FY 1974. The use of selected multiyear funding, restricted early in FY 1975 but subsequently authorized, has permitted budget flexibility essential both within and between fiscal years. As a result of decentralized contract management operations accomplished in NIAID in FY 1972, it has been possible to negotiate contracts relatively late in the fiscal year and the staff of the NIAID, Contract Management Branch has consistently handled this heavy workload with commendable skill and dispatch.

While personnel ceilings have kept collaborative staff heavily and constantly involved in their respective program efforts, training continues to be encouraged as it can be worked in. The NIH STEP Committee was chaired in 1975 by Dr. George Galasso, Chief, Infectious Disease Branch and Dr. John Nutter, Chief, Research Resources Branch is now a member of the STEP Committee. A number of staff attended STEP modules held during FY 1975 and found the information useful and the atmosphere stimulating. Administrative and secretarial staff participated in training relating to areas such as procurement, clerical skills, writing skills, travel, budget, personnel and general management.

NIAID Collaborative Research programs served as assignments for the orientation and training of four Grants Associates during FY 1975: Dr. Ronald Hopkins, Dr. Robert Beall, Dr. David Badman and Dr. Michael Gross each had approximately one month assignments in IDB while Dr. Ronald Hopkins received preceptoral training throughout the year from Dr. Minners. In addition to preceptoral activities, Dr. Minners serves as a member of the Grants Associate Board.

Consonant with previous manpower utilization studies conducted in Extramural Programs, NIAID and in other NIH organizations, a survey team headed by Mr. Charles Myers and assisted by Edward Vaarwerke, a management intern, was initiated in December 1974. The team consisting of Collaborative Research program and administrative staff, and including a Contract Management Branch representative, has developed a preliminary report, and it is hoped that specific recommendations on improving personnel utilization will be forthcoming.

At the time of the preparation of this report there has been an increasing number of requests for contract-related documentation under the Freedom of Information Act. This has created a special problem during April and May when there is customarily a very heavy staff workload for the processing of contract renewals and new actions. This problem is further complicated by the short response time required under the Freedom of Information Act. If this continues to impose such an additional workload, it is our hope that the responses to these inquiries and the preparation of documentation could be centralized in order to minimize the time and effort by our already overtaxed staff.

Additional burdens have been placed on the executive secretaries of the two chartered Collaborative Research advisory committees. New rules for committee management require longer lead times and more detail for actions relating to committee membership and conducting the meetings. An increasing amount of time is spent justifying closing portions of meetings and in preparing the required documentation for appointment of each new committee member. While recognizing the value of inputs received from the advisory committees, we need some relief and this might be achieved by the provision of more centralized support. Further it is our impression that some of the requests by the personnel officer and committee management are redundant, e.g. forms HEW 410, NIH 567 and HEW 532 might be consolidated into a single form and save considerable staff time.

In response to a request from the Office of the Director, NIH the NIAID developed a program description for Extramural Programs and Collaborative Research Programs roughly in the form of a "Tree." The Collaborative Research programs are presented in this year's report in conformity with the approved "Tree" and this is reflected in both the program status reports and the tabulations of individual contracts.

Because the U.S.-Japan Program responsibility includes grants as well as contracts, an overview of grants sponsored research as well as contracts are included in the narrative portion of this report.

Highlights of Program Development and Scientific Progress

Allergic and Immunologic Diseases

In support of the Institutes broadened efforts in research on allergic diseases, and particularly in reference to the 17 grant-supported Asthma and Allergic Disease Centers, a contract-supported allergen research reagents program has been developed. These activities, managed through the Research Resources Branch have resulted in the production of allergen research reagents for the defined antigens of ragweed pollen and parasitic roundworms. Reagents will be forthcoming for the allergenic antigens of rye grass and various venemous bees. A certification laboratory has been established at the Mayo Clinic to provide independent verification of contractor-producer materials and data. The ragweed antigens so far produced may prove useful in clinical situations as well as in research laboratories. Contracts have also been awarded to produce reagents for the radioallegosorbant test (RAST) and for a standard immunofluorescent reagent for immunoglobulin M.

The essential thrust of the present Transplantation and Immunology contract program is the development, assay and application of technology and products designed to enhance the survival of transplanted organs in man. To accomplish this objective, three approaches are currently being implemented;
1) evaluation of histocompatibility matching in predicting organ graft survival; 2) evaluation of Rabbit Anti Human Thymus Globulin (RAHTG) in preventing renal allograft rejection and 3) determining in experimental

animals methods for circumventing the rejection phenomena. The goal of the histocompatibility matching study is to study at least 2,000 well documented kidney transplant recipients. As of the time of this report, data from approximately 1,000 patients have been accessioned. Two more years will be required to complete the accession and another two year follow-up on each entry. The RAHTG study has achieved its goal of 100 patient entries -- 50 treated with RAHTG and 50 placebo patients. A two year follow-up will be required on each patient. The studies on immune regulation in experimental animal models are not yet sufficiently along to expect significant results.

The clinical trials under way are supported by a network of supporting contracts designed to provide highly specific reagents for research and a 72-well preloaded tray containing types for 32 generally recognized HL-A antigens. One contractor conducts studies on the immunopathology and etiology of organ rejection and provides a number of kidney transplant centers with analysis of biopsy material and patient's sera. Program sponsored workshops and catalogs have enhanced the value of the HL-A reagents for both clinical studies and research purposes.

Infectious Diseases

The three contracts awarded in FY 1974 for developing *in vitro* cultivation systems for *Treponema pallidum* served to initiate the Institutes contract program in venereal disease research. Two new studies were initiated in FY 1975 to study the antigens of *Neisseria gonorrhea* and use this knowledge to develop a serologic typing system and ultimately to prepare immunizing antigens for experimental vaccines.

Collaborative Research contracts and interagency agreements on hepatitis research are firmly linked to NIAID Intramural activities and are closely coordinated with those of the Department of Defense, the Center for Disease Control, Veterans Administration and Bureau of Biologics (Food and Drug Administration) and the National Heart and Lung Institute. A prototype inactivated vaccine for Hepatitis B was tested in chimpanzees for safety, antigenicity and protection against challenge. The vaccine proved effective in protecting the vaccinated animals against this challenge, and plans are underway to expand these trials in chimpanzees as the next step in the development of a candidate vaccine for eventual human use. The studies of the efficacy of hyperimmune gamma globulin in preventing Hepatitis B following transfusion or needlestick exposure are nearing completion.

The Research Resources Branch has produced and is distributing reagents for hepatitis B, viz HB_sAg, subtypes adr, ayw and adw and antisera for the core of the Dane particle (HB_c Ag). Interim reagents for hepatitis A research are now available for use in immune electron microscopy.

The NIAID sponsored Molecular Anatomy Laboratory at Rockville is currently the focal point of both reagent and vaccine production and assay. The latter activities are undergirded by basic studies of HB surface (HB_sAg) and core (HB_cAg) antigens and the enzymes and nucleic acid associated with the Dane particle core.

The influenza and other viral respiratory programs have produced significant results during the past year. A study is underway to determine whether all or a portion of the genome of influenza virus, RSV, or parainfluenza type 3 virus persists in the cells of the respiratory tract of man either as viral RNA or as an integrated complementary DNA. If persistence is found, the safety of live attenuated vaccines of these viruses will have to be reconsidered.

Although the Hong Kong ts-1-E recombinant influenza A vaccine has been shown to be safe and immunogenic in adults, administration of the vaccine to seronegative children resulted in reversion of the ts phenotype. The instability of the ts-1-E marker has been a temporary setback in the Institute's efforts to produce a live, attenuated influenza vaccine. Plans are now being made toward the evaluation of another recombinant, Hong Kong ts-1-A.

An experimental inactivated influenza vaccine monospecific for the neuraminidase antigen has received extensive field trials in Buffalo, New York. Data are currently being analyzed to determine the protective effect of the vaccine during a subsequent influenza epidemic.

Antisera have been produced against all the recognized fifteen influenza virus hemagglutinins and contract activities are continuing to produce antisera against the eight neuraminidases, the ribonucleoproteins and matrix proteins. These reference reagents, which cover the spectrum of human, animal and avian influenza A viruses will provide researchers and epidemiologists with rapid and accurate means of characterizing new influenza virus isolates.

An influenza reference center was established to collect all available influenza strains and serve as a library of antigen types with the underlying purpose of trying to fathom the derivation of new strains pathogenic to man.

Studies at the Molecular Anatomy Laboratory in Rockville, Maryland resulted in characterization of spike and ribonucleoprotein antigens of RSV, the production of specific antisera and the development of a double-antibody-radioimmunoprecipitation (RIP) assay for RNP. Examination of paired sera from documented human RSV infections showed good correlation of the RIP test with CF and plaque reduction responses. Sensitivity of the assay equalled or exceeded that of the plaque-reduction test.

Other disease control activities of the Infectious Disease Branch include the development and field testing of a number of bacterial vaccines. A large-scale field trial of the meningococcal A polysaccharide vaccine and H. influenzae type b PRP vaccine is currently underway in Finland involving approximately 100,000 children ages 3 months to 6 years.

Clinical evaluation studies of polyvalent pneumococcal polysaccharide vaccines are currently in progress in North Carolina and California. Efficacy trials of an octavalent pneumococcal vaccine in otitis prone children are scheduled to start this summer.

A field trial of a formalin-inactivated Mycoplasma pneumoniae vaccine ([illegible]-IA) has been carried out during this fiscal year in 7,861 volunteers at the Parris Island Marine Base. Although a significant difference in disease between vaccinees and controls has already been seen, a final estimate of efficacy must await completion of serologic assays.

A field trial employing purified cholera toxoid began in Bangladesh in July 1974 and was completed in December. Short-term effectiveness was demonstrated, particularly in young children. There was no demonstrable benefit from two injections of toxoid over one. Since purified cell wall antigens and standard whole cell vaccine also offer only short-term protection, the problem of providing long term immunity through immunization is still unresolved. Plans now call for investigating possible loss of immunogenicity of toxoid vaccines through sartorius filtration. Results from studies in experimentally vaccinated and challenged dogs have encouraged this avenue of approach.

Preliminary data from studies of diarrheal disease in Brazilian children indicate that heat-labile enterotoxin from lactose fermenting organisms are found in approximately 50% of the affected children.

Studies on leprosy have focused on immunopathologic studies of the disease processes and improved methods of treatment. Preliminary findings suggest lepromatous leprosy may be associated with immunologic disturbances of both skin and glomerular basement membranes. Quite possibly, humoral or tissue factors may be present in patients with lepromatous leprosy that may alter the kenetics of polymorphonuclear leukocytes (PMN) responses to inflammatory stimuli. Studies on defective leukotaxis and inflamation in leprosy lesions demonstrated that leukotactic may be present in leprosy serum. These features of chemotactic inactivator factor may be responsible, at least in part, for impairment of cellular inflamatory responses in leprosy.

Studies on the chemotherapeutic value of DDS are continuing in Cebu, P.I., but it will be another year or possibly more before significant results are forthcoming. Surveillence of post-treatment leprosy patients among a Pacific Island (Pingalapese) population produced the conclusion that eradication of leprosy in a population by use of DADDS for prevention cannot be effective without simultaneous and continuous drug control of all bacilliferous cases in the population.

A potential resource of M. leprae and lepromin is being further developed with a new contract award for maintenance of a colony of armadillos which can be infected with the organism.

Excellent progress has been achieved in preparing transfer factor for possible clinical trials in treatment of lepromatous leprosy. Approximately 175 human doses are available and are ready for testing for activity in normal recipients.

Increasing interest in the role of malnutrition and suseptibility to infections is evidenced by the Workshop activity in the U.S.-Japan Program which is carried out in collaboration with the National Institute of Arthritis, Metabolism and Digestive Diseases and the National Institute of Child Health and Human Development. For example, a "Workshop on Immune Status in Malnutrition" was held in May 1975 and another series is planned to generate working papers to define possible new program activities. An award made in FY 1975 for a contract entitled "Interrelationships of Early Malnutrition, Immunologic Competence and Immunity to Measles" will be carried out in Nairobi, Kenya, Africa.

One of the highlights of program-sponsored research in schistosomiasis was the establishment of a cell line from embryos of the snail host, *Biomphalaria glabrata*. As a result, investigators are able to utilize these cell lines in studying the larval stages of *Schistosoma mansoni in vitro*. Also significant was the finding that *B. glabrata* snails have the potential for developing acquired specific resistance to trematode infection. Chemical modification of the thioxanthene class of schistosomicidal agents offers the potential for a drug which is safer and as effective as the parent compound.

It is now established that *Wuchereria bancrofti*, thought to be an exclusively human parasite, will mature in monkey hosts. This finding will make possible a wide variety of needed experimental studies.

The tuberculosis program continues to provide a variety of reference reagents including atypical strains of *Mycobacterium tuberculosis* and a special kit of reference antisera. A program-sponsored activity has produced fractions from cell filtrate and cell extract of *Mycobacterium tuberculosis*, H37Rv. These fractions are being tested for serologic activity by immunodiffusion and immunoelectrophoresis and for specificity by lymphocyte transformation and by skin testing in guinea pigs.

Two mycobacterial vaccines are under investigation in experimental animals: (1) BCG, administered by inhalation or intravenously and (2) Youmans' mycobacterial ribosomal vaccine. Rhesus monkeys immunized intravenously or by inhalation with BCG are afforded a significant degree of protection when challenged by an aerosol of virulent tubercle bacilli. Preliminary data obtained in guinea pigs inoculated with Youmans' ribosomal vaccine and later challenged by aerosol or intraperitoneally indicate that the vaccine might be protective. In other program sponsored research, the need for T-lymphocytes in the development of both sensitivity and immunity to tuberculosis was demonstrated in a mouse model.

Research on viral diseases in the U.S.-Japan program has included projects on rabies and selected arbovirus diseases. In rabies research, the Rabies Fluorescent Focus Inhibition Test (RFFIT) was shown to be highly reproducible and was proven of value in studying inactivation by beta-propiolactone of the rabies virus. A purified rabies glycoprotein has been extracted which appears immunogenic in hamsters, rabbits and mice.

In studies of acute dengue hemorrhagic fever and dengue shock syndrome it was demonstrated that surfaces of B lymphocytes contain dengue antigen-antibody complex. Complement activation appears to result from large amounts of circulating immune complexes.

Dengue has been isolated from monkeys in the Malaysian jungles. Current studies on the jungle cycle of dengue are focussed on vector identification. Variation in susceptibility of mosquito vectors of dengue from one generation to another provides the potential for biologic regulation by possibly replacing susceptible vector populations with more refractory strains.

The Antiviral Substances Program is currently supporting efficacy studies of several promising antiviral agents. As a result of studies which indicate that interferon may be effective against osteogenic sarcoma, there is increased interest in this antiviral substance. Work is proceeding to make production of purified exogenous human interferon more efficient and more economical. The Program is also collaborating with intramural staff on the development of a new and promising endogenous interferon inducer (poly I· poly C-lysine complex).

As a result of studies supported by the Infectious Diseases Branch to determine the safety and efficacy of iododeoxyuridine (IDU) and adenine arabinoside (ara-A) in herpes encephalitis, it was discovered that IDU lacked efficacy and was excessively toxic. Use of the drug has been abandoned in such cases although earlier it had seemed to show promise in scattered anecdotal reports. Efficacy studies of ara-A are continuing, however, and the drug looks promising. A potent new antiviral substance, disodium phosphonoacetate (PAA) has been developed by Abbott Laboratories and large-scale efficacy studies comparing PAA with ara-A in facial herpes simplex are currently being developed under IDB contract.

The arboviruses contract program was brought to near completion with the last few reagents now in the final stages of testing. Support for the working version of the Catalog of Arthropod-Borne Viruses of the World is terminating and this activity will be supported in the future by the Center for Disease Control. Publication of the Second edition of the formal catalog was, however, supported by the NIAID and this volume is expected to be available by the end of June 1975.

It has been determined that the Research Resources Branch should enter into a cooperative enterprise with the ATCC which will eventually result in the transfer of certain Research Resources Branch viral reagent collections to the ATCC. There are presently four collections of seed viruses and corresponding antisera ready for this cooperative effort: enteroviruses, rhinoviruses, arboviruses, and adenoviruses. When the viability/activity approaches minimal acceptable levels, the Research Resources Branch "certified stock" would serve as seed material for the preparation of replacements by the ATCC at no cost to the Research Resources Branch.

A contract to accomplish this first phase of reagent transfer is currently under negotiation.

IDB

GMB

I&CR

RRB

TIB

RESEARCH RESOURCES BRANCH

The need for well characterized reference reagents as an adjunct to research is currently well recognized. In support of this concept, the Research Resources Branch conducts a program for the production, testing, and distribution of a wide range of seed viruses, mycoplasmas, allergens and corresponding antisera to research scientists. The branch identifies reagents for which a critical need exists, has these produced through the contract mechanism, distributes them as reference reagents and disseminates information and technical advice on reagent characterization and use.

I. Asthma and Allergic Disease Centers - Allergen Reagents Program

During the final month of FY74 a contract was awarded to the Mayo Clinic to serve as an allergen certification laboratory. This facility will serve Research Resources Branch as a central source of certification data for bulk and packaged allergen reference reagents produced by other contractors. The contractor will provide immunological and biological activity data as well as chemical and biophysical characterizations. It is anticipated that over the span of the next few years the acquisition of allergen associated reference reagents will become an increased activity of the Research Resources Branch. The concept of utilizing certification laboratories independent from production contractors has been used with great success in other elements of the RRB program. A central independent facility will allow a ready source for verification of product specifications and acceptability of products. In addition, it is expected that the allergen certification laboratory will provide an expanded characterization of these materials that would be an inappropriate part of production contracts. This is particularly important in terms of establishing the in vitro and in vivo biological activities of these reagents.

Ragweed antigens E, K, and Ra3 and a polyvalent ragweed antiserum have been produced under prior year contracts. Their certification was accomplished by the laboratory at the Mayo Clinic during FY75 and the announcement of their availability is imminent. Research Resources Branch plans to acquire an IND for the ragweed antigens and to subsequently distribute them for clinical trials as well as in vitro testing.

A contract was awarded to Harbor General Hospital near the end of FY74 to produce anti-human IgE for use in Radioallergosorbent tests. This contract is continuing and completion of production is anticipated during the first quarter of FY76. The test is widely useful in both research and diagnosis of allergic disease and it is believed by many investigators that it may eventually replace skin testing as a diagnostic tool. It is anticipated that this reagent will be particularly useful to investigators in the Asthma and Allergic Disease Centers.

The delivery of 500 mg of the allergen Asc-1 from Ascaris suum by a contractor at the University of Western Ontario has been delayed. During the course of the contract a critical material was withdrawn from the market and this necessitated additional developmental research by the principal investigator. Delivery is now anticipated during the first quarter of FY76 of this antigen which produces high levels of IgE in laboratory animals.

A contract was awarded to Worthington Biochemical Corporation to extract and package the Group Antigens I, II, and III from 9 kilograms of ryegrass pollen. This is the second contractor to attempt this work; the previous work resulted in a termination of the contract when the contractor failed to comply with agreed procedures and was unable to produce acceptable products during pilot runs.

Research Resources Branch is currently acquiring whole venoms from honey bees, paper wasps, yellow jackets, yellow hornets, and white-faced hornets. During the coming year these will be packaged and distributed in support of research in the Asthma and Allergic Disease Centers.

Research Resources Branch is currently supporting efforts of the World Health Organization to establish an International Standard for fluorescently labeled anti-human IgM. Immunofluorescence is either the only method, the method of choice, or plays an important role in the diagnosis of a wide variety of human diseases. The establishment of this standard reference material will significantly improve the quality of working materials of central importance in the diagnosis of important congenital infections.

II. Infectious Diseases - Special Concerns

Viral Reagents Program

Enteroviruses - During FY75 seven additional Lim Benyesh-Melnick intersecting typing pools were certified and released for distribution. The total array of 15 pools now available aid in the rapid identification of this important group of viruses. The seed virus preparation produced by the CDC for Toluca-1 has been certified and released for distribution and the corresponding antiserum is still in production.

The certification laboratory for this group of viruses continues to be located at the California State Department of Public Health and in addition to certification testing is also involved in the identification of new types and testing related to the long term storage behavior of selected reagents.

Arboviruses - The one remaining producing contractor for this group, California State Department of Public Health, is rapidly nearing completion of the work. During the first quarter of FY76 it is expected that all reagents for this large program will have been produced. This will accomplish the primary program objective of making available seed virus and antibody preparations for arboviruses of demonstrated or potential importance to disease on the North American Continent. Further production activities are not anticipated for individual viruses by Research Resources Branch and the collection will contain 82 reagent sets.

The certification laboratory for this program is located at the Yale Arbovirus Research Unit and serves as an independent source of data for verifying production specifications and adding other pertinent data to fully characterize and catalog the bulk and packaged materials.

With this fiscal year, Research Resources Branch is terminating its support of the Catalog of Arthropod-Borne Viruses of the World. This mechanism of information exchange has proven to be of great value to arbovirologists around the world and beginning with FY76 this work will be supported by the CDC through its Fort Collins, Colorado laboratory. Research Resources Branch support in FY75 included funds to publish, in conjunction with the CDC, the second edition of the formal published catalog, and distribution is expected to begin in June of 1975.

Eight WHO Collaborative Centers for Arbovirus Reference and Research have been supplied with each of the 30 arbovirus grouping fluids. These are of great usefulness in the preliminary identification of virus isolates and the conservation of scarce, highly specific antisera used for final identification. Their use in this international program will expand the data for them. Completion of six additional grouping fluids will complete the array necessary to work with all viruses in the Catalog.

Production of three additional antisera for identification of arthropod blood meals is now complete and following their certification during the last month of FY75, distribution will begin. The three reagents complete the planned array of 13 antisera for this program element.

Rhinoviruses - The production and certification activities for the 89 serotypes in this program are now complete and the reagents are ready for distribution. Further production of reagents for this group is not planned.

Myxoviruses - A contract for the production of monospecific antisera for the known viral subunits of the influenza viruses of man and animals is continuing. Presently the contractor has produced antisera against all fifteen hemagglutinins and after their certification early in FY76 they will become available for distribution. Work is continuing on antisera against eight neuraminidases, the ribonucleoproteins and the matrix proteins. Upon completion of all the work, the reagents will permit the rapid and accurate determination of the antigenic makeup of influenza isolates.

A contract with Aldrich Chemical Company has resulted in the production of a second lot of the synthetic neuraminidase substrate 2-(3'-methoxyphenyl)-N-acetyl-α-neuraminic acid (MPN). Following independent certification which is now in progress the reagent will be distributed to qualified requesters. This substrate will make possible a direct colorimetric assay of this enzyme of importance in the study of influenza viruses and their antigenic variations.

Parvovirus & Adenovirus Associated Viruses - Through the cooperation of Dr. Hoggan, LVD, NIAID three remaining antisera and one seed virus preparation for AAV were produced during FY75. It is anticipated that these reagents will be certified for distribution early in FY76. Research Resources Branch does not anticipate additional production efforts for these viruses.

Hepatitis - Past activities by Research Resources Branch have resulted in the acquisition of an extensive collection of reagents for hepatitis B. The array of reagents currently being distributed are for the following antigens: HB_sAg/adw, HB_sAg/ayw, HB_sAg/adr, and HB_cAg.

In March of this year a large shipment of hepatitis B reagents was made to Paris, France for use in a workshop. The reagents were collaboratively assayed by an international group of investigators along with samples from their own laboratories. As a result of this workshop a new nomenclature for HB_sAg may emerge. The quality and specificity of the Research Resources Branch reagents were confirmed and some may be selected as prototype reagents for the new nomenclature.

A preimmune and convalescent chimpanzee antiserum for hepatitis A is now available for distribution. This reagent is useful as a reference material when conducting immune electron microscopy, immune adherance tests or complement fixation tests for this viral infection of great public health importance.

Processing and Distribution Program

In addition to the various program elements detailed above, Research Resources Branch also distributes reagents for mycoplasmas, herpesviruses, adenoviruses, coronaviruses, reoviruses, and interferons.

The processing contractor is the American Type Culture Collection and work is proceeding at an optimal rate. This contract is a key part in the Research Resources Branch program since the expertise of the processing laboratory is relied on to prevent degradation of reagent quality during final processing and packaging.

The Research Resources Branch reagent collection now includes over 750 individual reagents. The repository and distribution contract remains at Flow Laboratories, Rockville, Maryland and a tabular record of distribution by that facility since FY69 follows:

FISCAL YEAR	TOTAL TRANSACTIONS	TOTAL AMPS & VIALS DISTRIBUTED
1969	402	15,805
1970	474	26,242
1971	638	12,902
1972	572	21,801
1973	575	19,181
1974	500	9,932
1975 (9 months)	442	4,790

During the first three quarters of FY75 the distribution of reagents by groups is shown in the following table.

During this past year the Research Resources Branch staff has been involved in the preparation of the 1975 Catalog of Research Reagents. The catalog is scheduled to be sent to the Government Printing Office for printing the first part of June and will probably be available in the fall for mailing to over 1,000 laboratories and scientists currently on the catalog mailing key. The new catalog will greatly increase the value of the reagents by supplying new and updated data as well as describing recently developed reagents for the first time. It is anticipated that the new catalog will result in an increased number of requests for reagents during FY76.

It is generally understood that Research Resources Branch should be involved in supporting those areas of research that are of current scientific interest and especially in those that need support for establishing defined reference reagents. After a category of reagents has become established and the area of research that it supports well defined it is of questionable value for Research Resources Branch to continue storing and distributing that category of reagents for a prolonged period of time. This is especially true when other organizations can be identified that have as their main objective the distribution of such materials to the scientific community. The American Type Culture Collection is such an organization and there is an area of overlap with materials that Research Resources Branch distributes, namely some virus seed materials.

It has been determined that Research Resources Branch should enter into a cooperative enterprise with ATCC which will eventually result in the transfer of certain Research Resources Branch viral reagent collections to the ATCC. There are presently four collections of seed viruses and corresponding antisera ready for this cooperative effort: enteroviruses, rhinoviruses, arboviruses, and adenoviruses.

When the viability/activity approaches minimal acceptable levels, the Research Resources Branch "certified stock" would serve as seed material for the preparation of replacements by the ATCC at no cost to RRB.

Research Resources Branch will award a contract to the ATCC during the last month of FY75 to effect this transfer of reagents.

RESEARCH RESOURCES BRANCH
DISTRIBUTION OF SPECIFIC VIRUS GROUPS
July 1, 1974 - March 31, 1975

# of Items Avail. (% of Inventory)	Virus Group	SHIPMENTS # of	SHIPMENTS % of	AMPOULES # of	AMPOULES % of # Distributed
62 (8.4)	Adenovirus	11	2.5	148	3.2
154 (20.9)	Arbovirus**	23	5.2	247	5.3
229 (31.0)	Enterovirus***	64	14.5	982	21.2
6 (.8)	Hepatitis	152	34.5	964*	20.8
15 (2.0)	Herpesvirus	11	2.5	28	.6
5 (.7)	Interferon	50	11.3	88	1.9
55 (7.5)	Mycoplasma	31	7.0	350	7.5
63 (8.5)	Myxovirus Paramyxovirus and related	28	6.4	157	3.4
4 (.6)	Ragweed	19	4.3	65	1.4
6 (.8)	Reovirus	7	1.6	44	.9
139 (18.8)	Rhinovirus	6	1.4	138	3.0
	Combinations	39	8.8	1429	30.8

Figures based on 441 shipments and 4,640 ampoules shipped (excludes testing, packaging, and WHO).

* Does not include 1 shipment totaling 150 ampoules to Paris for hepatitis workshop.
** Includes 14 shipments of Grouping Fluids totaling 229 vials.
*** Includes 45 shipments of LBM typing pools totaling 815 vials.

Molecular Anatomy Program

The major objective of this program is the development of methods for isolating viruses and viral antigens and the application of those techniques to research on associated diseases. This program is a cooperative project of NIAID and the U.S. Energy Research and Development Administration. The Rockville Laboratory of the Molecular Anatomy Program (MAN) is currently in its sixth year of operation. The proximity of this facility to NIH is of great benefit in fostering close collaboration between the scientific staff of MAN and the staff of NIAID. The training of NIAID intramural scientists and contractors in the research application of specialized instrumentation is also an important aspect of the laboratory's mission.

An expansion of the Rockville Laboratory which was approved earlier was completed during FY75 and added approximately 2200 square feet of space. The laboratory containment facilities were also expanded and both improvements will yield a return in future years. Maximum benefit will be realized by projects involving biohazards and the improvements are currently facilitating expanded research on hepatitis with maximum safety.

During FY75 NIAID made the formal decision to proceed with the development of a vaccine for hepatitis B. The decision was based on preliminary results from chimpanzee experiments using the 22 nm form of HB_sAg purified from the serum of a chronic carrier as the immunizing antigen. The vaccine did not cause disease, it stimulated humoral antibody and protected animals against disease following experimental challenge with live virus of the homologous subtype. A "second-generation" vaccine is presently being prepared in the new containment facilities of the MAN Program. The logistics of a program of this size required the redirection of funds to supplement the equipment, material, and technical staff. A new centrifuge and zonal rotor were purchased and installed and an additional technician was hired and trained for zonal centrifuge operation. The first sublot of HB_sAg vaccine, subtype adw, is being made from a pool of individual units obtained by plasmapherisis of chronic antigen carriers. Multiple units from each individual were pooled and aliquots combined to represent the bulk starting material. Extensive safety testing of the starting material was performed and the starting bulk material passed all safety tests including those for microbial sterility and adventitious viral agents. The vaccine is currently being purified by an established protocol involving zonal centrifugation procedures.

The inventory of adsorbed guinea pig sera (anti-HB/ad), prepared several years ago for distribution by the Research Resources Branch was almost depleted during FY75. Therefore, at the request of Research Resources Branch, the contents of three thousand vials of wet-frozen unadsorbed sera from the original production schedule were thawed, pooled, and processed as before. Antibodies to normal human serum components were removed by an affinity chromatographic technique. The processed serum pool was free of antibodies to normal serum and excellent recovery of specific anti-HB_s/adw was obtained. The pool was adjusted to approximate the titer of the original antibody, sterile-filtered, and vialed. This reagent is now available for distribution.

Other studies on hepatitis B were conducted during FY75 including the immunochemistry of the structural polypeptides of HB_SAg and the antibody response of laboratory animals. Detailed studies continued on the mechanism of DNA replication associated with the HB_SAg-specific polymerase as well as a characterization of the polymerase-containing Dane particles.

Studies with the Respiratory Syncytial virus were focused on the characterization of the antisera to the spike and ribonucleoprotein (RNP) antigens and the development of a double-antibody radioimmunoprecipitation (RIP) assay for RNP. The antisera to spike and RNP were evaluated by indirect fluorescent antibody techniques and infected HeLa cells were examined with these antibodies using both live and fixed cells. Anti-RNP fluorescence was observed with fixed cells but not live cells indicating that it was an internal antigen, while anti-spike serum stained both fixed cells and the membranes of live cells. Different patterns of fluorescence were observed with these antisera during the time course of infection. Paired sera from well documented cases were obtained and assayed by RIP for RNP. In all cases a rise in antibody to RNP was observed to correlate with rises in both CF and plaque reduction antibodies. The sensitivity of the assay equaled or exceeded that of the plaque-reduction test.

Research and development at Oak Ridge in support of the Rockville Laboratory pertains to improvement of the various apparatus applications, construction of safety devices for biohazardous operations, and investigation of automated systems for eventual transfer to the daily operations of the MAN Program.

INDIVIDUAL CONTRACTS AND AGREEMENTS TABULATION
RESEARCH RESOURCES BRANCH

Allergen Reagents Program

Contract/ Ag. No.	Contractor Principal Investigator	Objectives & Findings	FY75 Obligations FY76 Plans
N01-AI 42546	Mayo Clinic Gleich, Gerald	This facility serves as the certification laboratory for the Allergen Reagents Program. Bulk products are assayed to ascertain if they meet contract specifications and final packaged materials are tested for potency and are fully characterized	Prior Year Support Continuation
N01-AI 42547	Harbor General Hosp. Heiner, Douglas	To produce an anti-human IgE antiserum for use in the radioallergosorbant test for assaying the allergic status of individuals	$10,000 Expiration
N01-AI 42500	Univ. Western Ontario Strejan, Gill	To produce 500 mg of the allergen Asc-1 from *Ascaris suum*.	$7,000 Expiration
N01-AI 52516	Worthington Biochem. Corporation Flora, Robert	To extract and package the group antigens I, II, and III from 9 kilograms of ryegrass (*Lolium perenne*) pollen.	$54,000 Expiration
N01-AI 52503	World Health Org. Goodman, Howard	To acquire in collaboration with the International Union of Immunological Societies an International Standard reagent for fluorescently labelled anti-human IgM	$20,000 Expiration

Viral Reagents Program

Contract/ Ag. No.	Contractor Principal Investigator	Objectives & Findings	FY75 Obligations FY76 Plans
N01-AI 90089	Calif. State Dept. of Public Health Schmidt, Nathalie	This laboratory serves as a certification facility for enteroviruses. In addition to certification testing of bulk and final packaged materials produced by other contractors it is also involved in the identification of new types and storage stability tests.	$39,000 Expiration
3-Y01-AI 40004-02	Center for Disease Control Suggs, Morris	To produce a seed virus preparation and immune and control sera for the enterovirus Toluca-1	$12,000 Expired
N01-AI 80679	Cal. State Dept. of Public Health Emmons, Richard	The contractor is the sole producing contractor for arboviruses. During this terminal contract period he is producing reagents for Dengue 1 and 4, LCM, Bocas and Gumbo Limbo viruses.	Prior Year Support Expiration
N01-AI 42517	Yale University Shope, Robert	This laboratory is the certification facility for the arboviruses. All reagents in this category are assayed in bulk and final packaged form to assure that they meet contract specifications, are potent, and completely characterized.	$115,000 Continuation

Viral Reagents Program (Cont'd)

Contract/ Ag. No.	Contractor Principal Investigator	Objectives & Findings	FY75 Obligations FY76 Plans
2-Y01-AI 20007-05	Center for Disease Control Berge, Trygve	This facility registers data on new arboviruses and disseminates this information at quarterly intervals to an international array of participating scientists. Also collects and distributes information concerning registered viruses from published materials and other sources. At the end of FY75 will publish a second edition of the Catalog of Arthropod-Borne Viruses of the World.	$46,000 Expired
N01-AI 70059	Smithsonian Inst. Handley, Charles	Contractor served as a central resource for identification of mammalian species submitted by laboratories involved in ecological studies related to viral diseases with special reference to arboviral diseases.	Prior Year Support Expired
N01-AI 32512	Univ. of California Tempelis, Constantine	Contractor has produced antisera against blood meals of arthropods important as vectors of arboviruses. During this terminal period antisera specific for reptilian, amphibian and mammalian meals were made.	Prior Year Support Expired
N01-AI 42521	Florida Dept. of Hlth. Edman, John	Serves as the certification laboratory for arthropod blood meal antisera	$7,000 Expiration

Viral Reagents Program (Cont'd)

Contract/ Ag. No.	Contractor Principal Investigator	Objectives & Findings	FY75 Obligations FY76 Plans
N01-AI 12111	Univ. of Wisconsin Dick, Elliot	Single remaining producer of rhinovirus reagents and during the terminal period produced reagents for five serotypes.	Prior Year Support Expired
N01-AI 71188	Univ. of Vermont Phillips, C. A.	Served as the rhinovirus certification laboratory and during the total term of the contract tested both seed virus and antiserum preparations for all 89 serotypes in this collection.	$12,000 Expired
N01-AI 32516	St. Jude Hospital Webster, Robert	This contractor is producing monospecific antisera against all the known subunits of the influenza viruses of man and animals. At this time he has produced antisera for all 15 hemagglutinins and two neuraminidases	$54,000 Expiration
N01-AI 42526	Aldrich Chem. Co. Warawa, Edward	Produced the synthetic neuraminidase substrate 2-(3'-methoxyphenyl)-N-acetyl-α-neuraminic acid. This reagent facilitates assay of this enzyme which helps characterize influenza viruses.	Prior Year Support Expired
N01-AI 42544	George Washington Univ. Zook, Bernard	Served as a guinea pig holding facility during the production of antisera for three AAV serotypes.	$2,000 Expired

Processing and Distribution Program

Contract/ Ag. No.	Contractor Principal Investigator	Objectives & Findings	FY75 Obligations FY76 Plans
N01-AI 42515	American Type Culture Collection Stevens, David	This contractor processes and packages bulk reagents produced by other contractors. A single facility assures that all reagents are uniformly packaged with the same level of quality	Prior Year Support Renewal
N01-AI 22515	Flow Laboratories Adkins, Harry	Serves as a repository and distribution facility for reference reagents	$161,000 Renewal

Molecular Anatomy Program

Contract/ Aq. No.	Contractor Principal Investigator	Objectives & Findings	FY75 Obligations FY76 Plans
2-Y01-AI 00002-07	Energy Research and Development Admin. Gerin, John	The major objective is to develop and apply methods of isolating viruses and viral antigens and the application of those techniques to research on associated diseases. During FY75 this laboratory was involved in a variety of studies on hepatitis B but the most significant involvement was the central role of this facility in developing a hepatitis B vaccine. Application of techniques developed during prior years are being applied in this project. Other project areas investigated were: hepatitis B reagents, immunochemistry of hepatitis B polypeptides, isolation and characterization of antigenic components of respiratory syncytial virus, studies on acute infectious nonbacterial gastroenteritis and hepatitis A as well as serving NIAID as a training facility for intramural scientists in selected techniques.	$479,000 Renewal

Part A - Contractors Publications

Chisari, F.V., J.L. Gerin, and T.S. Edgington. "Immunochemistry of the Hepatitis B Virus: ^{125}I HB Ag Ligand." J. Immunol. 113:543-553, 1974.

Feinstone, S.M., A.Z. Kapikian, J.L. Gerin, and R.H. Purcell. "Buoyant Density of Hepatitis A Virus-Like Particles in Cesium Chloride." J. Virol. 13: 1421-1414, 1974.

Gerin, J.L. "Sturcture of Hepatitis B. Antigen (HB Ag)." In: Mechanisms of Virus Diseases. W. S. Robinson and C.F. Fox (eds.). Menlo Park, Cal.: W.A. Benjamin, Inc., 1974.

Gerin, J.L. "The Dane Particle in Type B. Hepatitis." In: Transmissible Disease and Blood Transfusion, Sixth Annual Red Cross Scientific Symposium, G. A. Jamieson (ed.). New York: Grune & Stratton, in press.

Gerin, J.L., R.M. Faust, and P.V. Holland. "Biophysical Characterization of the adr Subtypes of Hepatitis B Antigen and Preparation of anti-r Sera in Rabbits." J. Immunol, in press.

Gerin, J.L. J. W-K. Shih, and P.M. Kaplan. "Biophysical and Biochemical Characterization of Hepatitis B. Antigen." In: National Academy of Sciences Symposium on Viral Hepattits. S. Krugman (ed.). Amer. J. Med. Sci., in press.

Gerin, J.L. and D.D. Willis. "Absolute Containment of the Preparative Ultracentrifuge in Hepatitis Research." In: National Cancer Institute Symposium on Biohazards, Cancer Research Safety Monograph Series, Vol. 1, p. 95. L.S. Idoine (ed.). Litton Bionetics, Frederick, Maryland, 1973

Hoffman, E.J., E.C. Ford, and J.L. Gerin. "Isolation and Characterization of Ribonucleoprotein of Respiratory Syncytial Virus.: Abstr. ASM Ann. Mtg., April 1975.

Kaplan, P.M., J.L. Gerin, and H.J. Alter. "Hepatitis B-Specific DNA Polymerase Activity During the Course of Post-Transfusion Hepatitis." Nature, 249: 762-763, 1974.

Karabatsos, N. "Antigenic Relationships of Group A Arboviruses by Plaque Reduction Neutralization Testing." Am. J. Trop. Med. & Hyg., 24: 527-532, 1975

Purcell, R.H., and J.L. Gerin. "Hepatitis B. Subunit Vaccine: A Preliminary Report of Safety and Efficacy Tests in Chimpanzees.: In: National Academy of Sciences Symposium on Viral Hepatitis, S. Krugman (ed.). Amer. J. Med. Sci., in press.

Shih, J. W-K., P.L. Tan, and J.L. Gerin. "Immunochemistry of HB_sAg Polypeptides.: Abstr. ASM Ann. Mtg., April 1975.

Webster, R.G., V.A. Isachenco, and M. Carter. "A New Avian Influenza Virus from Feral Brids in the USSR. Recombination in Nature?" Bull. Wld. Hlth. Org., in press.

Part B - Staff Publications

None

Part C - Workshops

Research Resources Branch sponsored a workshop on Bunyaviruses during December, 1974. The major emphasis was on the basic virology of the viruses in this taxon which includes some important human pathogenic arboviruses. The workshop presented a comprehensive review of the status of research including antigenic interrelationships, vector relationships, biologic variety and human disease problems. RRB does not anticipate further work on members of this group in the near future.

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TRANSPLANTATION AND IMMUNOLOGY BRANCH

During fiscal year 1975, approximately 2.2 million dollars were obligated on Transplantation and Immunology Branch contracts and agreements. The goals of the contract program are to develop and coordinate methods, procedures, and resources to enhance research in transplantation and immunology and to develop and utilize new methods for the evaluation and monitoring of such programs.

I. Clinical Immunology - Transplantation and Immunology Contracts

1. REAGENTS AND TECHNIQUES PROGRAM

Since its inception in 1965, much progress has been made in the development of methodology for histocompatibility typing and in making quality reagents available to worldwide research laboratories. Seven contracts provide funds to: compare various tissue typing techniques; develop new procedures that are simpler and more accurate than those currently employed; produce a continuing supply of high quality histocompatibility typing sera; conduct research directed at discovering new antigen specificities; and to provide other reagents needed in immunology research.

Refinements in typing techniques have been developed and evaluated by three contractors. Techniques for histocompatibility antigen cross-matching are being evaluated to determine the most effective method to eliminate the problem of hyperacute kidney rejection. Automated procedures are being evaluated for the fluorochromasia and platelet complement fixation tests. Procedures have been developed for the serological detection of histocompatibility antigens formerly only recognized by the mixed lymphocyte culture technique. The evaluation of the importance of non HL-A histocompatibility antigens in renal and bone marrow transplantation is continuing. The discovery of new loci of human histocompatibility recognition further clarified the complexities of tissue matching. Neutrophil specific antigens are being studied and their role in bone marrow transplantation is under evaluation.

The NIAID typing Serum Bank currently contains over 1,600 sera with a total volume of 278 liters. Additions to the Bank in FY'75 include 87 sera obtained by contracts from two laboratories, 69 donated by tissue typing laboratories, and 66 purchased outright by the Branch. Seven typing laboratories performed quality control studies on the sera prior to their acquisition, thereby assuring the reliability of Bank reagents. This was due, in part, to the increase in the numbers of worldwide solid organ transplants, very considerable demands are being made on the Bank for reagents currently being used in studies of the relationship between HL-A antigens in a variety of diseases including: ankylosing spondylitis, psoriasis, a number of allergic conditions, multipile sclerosis, chronic hepatitis, and malignant diseases. The demonstration that white blood cell and platelet survivals are markedly increased with donor-recipient matching has prompted the initiation of a number of programs to type blood bank donors. Because of the number of patients involved, a system of rationing reagents for this purpose will have to be imposed. The Branch is cooperating with blood banks in providing advice and limited supplies of reagents to permit them to develop their own programs to produce typing reagents.

Through the use of standardized trays and uniform lymphocytotoxicity typing techniques, a continuing effort is being made to improve the quality of reagents and the accuracy of laboratory results. Over 24,000 trays were provided to 125 U.S. laboratories for typing for solid organ and bone marrow transplantation. During the past year significant improvements have been made in the quality of reagents used in these trays so that laboratories may now type for 32 histocompatibility antigens.

Procedures have been developed to produce and distribute trays containing human lymphocytes with well-characterized HL-A antigens. These trays, now being field tested, will be used as sources of standard antigens to measure preformed antibodies in potential transplant recipients and as standards for the testing of new antisera.

A contract has been awarded for the production of HL-A Xenoantisera. By immunizing rabbits with human antigen material, sera are being produced which may be used for typing. This procedure will provide a new source for scarce, rare specificity antisera.

Other immunological reagents, produced by TIB contractors, including human complement components and inhibitors, and mouse H-2 antisera and soluble antigens are being distributed to qualified investigators. Since a large percentage of NIAID TIB Serum Bank users are NIH grantees and contractors, this program provides a very direct benefit to a number of NIH supported investigators. The continuing availability of these otherwise non-obtainable reagents makes it possible for them to continue their research.

2. EVALUATION OF TISSUE MATCHING IN RENAL AND BONE MARROW TRANSPLANTATION

Although histocompatibility matching has been shown to improve the prognosis on living related kidney transplants, no conclusive data have been obtained concerning the importance of this procedure in cadaver transplants. Since cadavers are the principal source of organs for transplantation, and since a principal impediment to transplantation is the non-availability of "matched" kidneys, it is important to determine the importance of HL-A antigens as soon as possible. Preliminary reports from a number of programs are suggesting that matching is important, most of these deal with small numbers of transplants with a relatively short follow-up. A very large Government investment is being made in providing reagents, services and patient care funds for approximately 3,200 U.S. transplants a year. Little reliable data is available concerning tissue typing and the role that tissue matching plays in the survival of their transplanted kidneys. The Advisory Transplantation and Immunology Committee of the NIAID has recommended that a program be established to collect complete and accurate clinical and laboratory data on renal transplant patients, to determine the efficacy of tissue matching.

At the present time contracts have been awarded to 16 regional transplant centers involving 36 hospitals and a statistical-data management center to participate in this study which will acquire approximately 800 new patients yearly. Uniform data collection forms are being used, by well-trained and supervised transplant registrars, to collect information which is forwarded to the statistical center. All laboratories in the study use uniform procedures and frequent checks are made to assure their continuing proficiency in tissue typing. Periodic training and testing sessions are held where participating laboratories are evaluated and instructed in new techniques. Committees of the participating investigators have been formed and a Policy Advisory Board has been appointed to provide all supervision of the program. Since January 1, 1974, 1,075 patients have been started on the study. By the end of 1976, approximately 2,000 well-studied kidney transplants will be entered. These patients will be followed for a minimum of two years, at which time statistical analyses will be made of the kidney and patient survivals of various categories of patients.

Bone marrow transplantation has been shown to be a promising treatment for a number of hematologic, neoplastic, and immunologic diseases. The key to clinical success lies in the understanding and control of the immunologic factors involved in rejection and graft versus host disease. Techniques being utilized in these studies include, HL-A serotyping, genetic studies, mixed lymphocyte culture, cell-mediated lympholysis, and antiglobulin reactions.

3. ANTILYMPHOCYTE GLOBULIN PROGRAM

Funds are provided for a double-blinded study of rabbit anti-human thymus globulin (RAHTG). One hundred cadaver kidney transplant recipients were treated with either RAHTG or a rabbit globulin placebo in a study which is designed to determine the efficacy of the RAHTG in preventing renal allograft rejection. The material used has demonstrated efficacy in prolonging skin and renal grafts in subhuman primates. This is a joint study with the Bethesda Naval Medical Research Program which developed the RAHTG and has the IND for its clinical evaluation, and the University of California, San Francisco where the clinical trial was conducted. The RAHTG used was developed with funds provided to the Navy by the TIB and animal testing was conducted at Bionetics Laboratories under an NIAID contract. All patients have now been entered on study and are now in the two-year follow-up period which will study long-term survivals and toxicity. This study is the first to use the double-blinded technique to evaluate an ALG produce of proven safety and efficacy in subhuman primate testing. The use of a single institution for the clinical trial limits the number of variables seen in many such studies.

4. REGULATION OF IMMUNE RESPONSIVENESS

During the past ten years, the Transplantation and Immunology Branch contract program has been directed primarily toward drugs and histocompatibility matching as a means for preventing or overcoming graft rejection. Graft rejection may be prevented by careful selection of the donor to avoid introducing a foreign antigen into the recipient. The chances of finding a perfect match when using a cadaveric organ is extremely poor. The numbers of four antigen matches for cadaveric donor transplants will hopefully increase by standardization of reagents through the NIH Tray Program and the proliferation of organ exchange programs. However, the problem of rejection in the mismatched transplants will remain.

In an effort to circumvent this problem of rejection, several programs were iniated; namely chemo-immunosuppressive drug evaluations, biological immunosuppressive effectiveness (antilymphocyte serum or globulin) and "immunological tricks" or manipulation of immune response by altering foreign antigen recognition and thereby selectively suppress the cellular and humoral immune response. Current drugs, while useful, are very toxic, producing marrow depression and therefore pan-immunosuppression. Chronic administration of steriods in high doses often produces the attendant complications of hypercortisonism. A controlled study of antilymphocyte globulin is underway and should help clarify its role as a biological immunosuppressant (Part 3).

After reviewing the state of the art, the Transplantation and Immunology Committee recommended the establishment of a contract program to investigate new methods of regulating immune responsiveness. Three contracts were awarded as a pilot effort in this field. One contract is investigating the rat uterus as an immunologically privileged site to ascertain the immunological mechanisms involved in fetus term and postpartum survival who bear alloantigens incompatible with their mother's antigens. Another contract is studying the mechanism of certain activated thymus(T) cells to regulate the response of normal thymus cells to alloantigens. These regulator T cells may either stimulate or suppress the immune response and may control its intensity. The final contract in this area is studying the immunosuppressive effect of alpha-globulin serum fractions. Thus, this interesting area of immune regulation is being studied from three sites of immunological reactivity; the organ, serum and cellular locations.

5. IMMUNOPATHOLOGY PROGRAM

Since the major course for concern in renal transplantation is organ rejection, the Transplantation and Immunology Branch has since 1968 provided immunopathologic diagnostic facilities for transplant programs throughout the United States. Renal tissue and/or sera from nearly 3,600 patients have been submitted for immunopathologic study since the inception of this program. During the past year, 714 renal and other organ biopsy specimens, and 717 serum samples from 869 patients were submitted by 76 institutions for study. Immunofluorescent, radio-immunoassay and antigen elution studies are used to detect various antibody, immunocomplexed and complement deposits on renal glomeruli and tubules. In addition, these specimens are tested for various etiologic agents responsible for the diseases of kidneys.

These procedures have contributed significantly to the understanding of kidney diseases in many nephritic patients throughout the country. The contractor has been able to differentiate immunopathologically the mechanism of graft failure, i.e. whether failure is due to a recurrent nephritis the deposition of circulating immune complexes in the renal glomeruli or tubules, or to an immunologic histoincompatibility reaction. This has facilitated the diagnosis and treatment of patients with renal failure.

Extensive efforts are currently being employed to ascertain whether these various immunopathological tests can predict graft rejection in the transplanted patient.

6. PUBLICATION AND CONFERENCE SUPPORT

As part of its information program, the Branch prepares an annual updating of the Catalog of Tissue Typing Antisera. The current issue of this Catalog was distributed to approximately 500 serum users in November 1974. The 920 page Catalog lists the characteristics of over 1,600 sera together with information submitted from over 200 users. This includes reports of specificity, utility, titers, and frequency of positive reactions for various racial groups. This is the only such document available and is used throughout the world by transplantation immunologists, geneticists, hematologists, and scientists investigating the association of HL-A antigens in a variety of diseases.

The Manual of Tissue Typing Techniques, revised this year, contains current information on the various procedures used in tissue typing, and was distributed to laboratories and used in tissue typing courses. The Branch also distributes catalogs of other reagents, including H-2 (mouse) antisera, complement components, and soluble antigens.

A TIB contract provided partial support for the planning and conduct of the International Histocompatibility Workshop, held at Aarhus, Denmark in June 1975. Funds were also allocated for the American Society of Clinical Histocompatibility Typing meeting held in Birmingham, Alabama in April 1975. Following this, a workshop was held to provide for training in the preparation of lymphocytes for typing, cell freezing and the use of microcytotoxicity typing techniques. Laboratories participating in the NIAID evaluation of tissue matching program participated in this two-day workshop which was designed to further improve the quality of typing in this study.

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Reagents and Techniques   Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY'75 Obligations FY'76 Plans
AI-81298	Cleveland Clinic Braun, W.E.	Establish and operate a regional leucocyte phenotyping facility. To evaluate tissue typing techniques, to match organ donors and recipients utilizing microcytotoxicity typing and cross matching procedures, to provide 5000 ml of typing antisera to the NIH Bank, and to provide consultative services to immunology laboratories. The contractor acquires and maintains a large reference panel of frozen cells which are well characterized. This panel is used to test the quality of sera being obtained for the NIAID Serum Bank, and to test the NIAID Serum Trays. The micro mixed lymphocyte culture, cross match, cell mediated lympholysis, and immune globulin techniques are used to ascertain the degree of donor-recipient immunologic identity that exists in human kidney transplants.	$107,040 Renewal
AI-42508	University of Leiden van Rood, J.J.	Search for anti-histocompatibility sera and improvement of techniques for testing. To operate a large program to screen human sera and to obtain, characterize and forward to the NIAID sera found to contain histocompatibility antibodies. To compare and evaluate various techniques for detecting histocompatibility antibodies. This program produces approximately 5,000 ml of much needed, reagent quality, antisera which is distributed through the Serum Bank to other investigators. New	$ 96,556 Renewal

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Reagents and Techniques Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY'75 Obligations FY'76 Plans
AI-42508	University of Leiden van Rood, J.J. Continued	automated techniques are being compared. Information obtained will help in the standardization of reagents and materially improve the accuracy of histocompatibility typing.	
AI-32508	Biomedical Research Inst. American Foundation for Biological Research Perry, V.P.	Freeze-drying of typing sera and processing, freezing and quality control of lymphocyte cells. To freeze-dry typing sera in a manner which completely preserves the typing reactivity of the reagent; in addition BRI is to develop a procedure to freeze, well-characterized lymphocytes in a multiwell tray which can be used as a uniform source of antigens. During FY'75 approximately 108 liters of antisera were freeze-dried in 1 ml vials. All bulk shipments are now sent at ambient temperatures. This has resulted in a considerable savings for an average shipment. This laboratory has determined the activity of over 1200 freeze-dried sera during the past year. BRI is continuing the research and development of a frozen cell tray from lymphocytes which have been (1) frozen and thawed and (2) stored in nutrient medium before plating and freezing. Other laboratories are cooperating by testing the frozen cell trays after shipping in dry ice.	$133,585 Renewal

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Reagents and Techniques   Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY'75 Obligations FY'76 Plans
AI-22515	Flow Laboratories Adkins, H.	Maintain a supply repository for processing and distribution of NIAID reagents and serum. To provide laboratory and warehouse facilities to process, store and ship reagents. This contractor made 1,341 shipments of bulk antisera and NIH tissue typing trays to 329 world-wide investigators. Fifty-two liters of antisera were received and processed.	$159,471 Renewal
AI-42557	Scripps Clinic and Research Foundation Ferrone, S.	Develop the best experimental conditions for the production of monospecific HL-A xenoantisera. An operationally monospecific W24, developed by the contractor, is being used on the NIH tray. The principal investigator is trying to reproduce his results using the same (1) donors serum as a source of soluble Ag, (2) dosage schedule and (3) host (rabbit). Other soluble antigen preparations, dosage schedules and hosts are being investigated as possible alternatives in the development of the best method to produce monospecific HL-A xenoantisera.	$ 48,716 (2 year) Renewal

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Reagents and Techniques Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY'75 Obligations FY'76 Plans
AI-12443	Microbiological Associates, Inc. Trapani, R.J.	Preparation and evaluation of human histocompatibility typing trays. To produce and package for delivery, serum and cell typing trays for human histocompatibility typing techniques standardization. Approximately 50,000 serum typing trays have been used by 125 tissue immunologists in determing recipient and donor HL-A transplant antigens. The results of their typings are statistically comparable and aid in organ exchange programs and analysis of the efficacy of histocompatibility matching in organ graft survival.	-0- (prior year) Renewal
AI-12257	Jackson Laboratory Cherry, M.	Mouse alloantisera production, storage and distribution. To produce, store, quality control and distribute to qualified investigators, H-2 mouse alloantisera. At least 211 investigators have used these antisera for basic research in the immunogenetics of cellular and humoral immunity, graft rejection, tissue compatibility, tissue disease degradation and oncogenesis in the mouse model which is analogous to the HL-A tissue antigen system in humans.	-0- (prior year) Renewal

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Evaluation of Tissue Matching in Renal and Bone Marrow Transplantation Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY'75 Obligations FY'76 Plans
AI-32511	University of Washington Thomas, E.D.	Evaluation of Tissue Typing Techniques in Bone Marrow Transplantation to determine the importance of histocompatibility matching in bone marrow transplantation. This contractor does more marrow transplants than any other center. The importance of matching in the prevention of severe graft versus host disease and on the eventual outcome of the transplant is studied in living related transplants. Data to date indicates that histocompatibility identical matches with living related donors do better than mismatched bone marrow transplants. Also being investigated is the effect of transplantation on the immune responsiveness of the recipient.	$ 71,389 Renewal
AI-50001-00	Naval Medical Res. Inst. Homer, Louis D.	Operation of a Statistical Data Management Center. Collection and analysis of data from 16 transplant centers on approximately 850 kidney transplants per year. This program has been operational since January 1974 and data has been collected on all transplants done in participating hospitals since that time.	$296,454 Renewal

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Evaluation of Tissue Matching in Renal and Bone Marrow Transplantation Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings			FY'75 Obligations FY'76 Plans
		Evaluation of histocompatibility matching in renal transplantation. Provide clinical and laboratory data to statistical center on all kidney transplants done in participating hospitals. Maintain quality control of matching procedures, freeze and store cells to allow subsequent immunologic studies. In most cases, data collection began January 1, 1975.			
		# of Transplants	# of Hospitals	# of Laboratories	
AI-22529	University of Illinois Jonasson, Olga	120	7	1	$ 79,595 Renewal
AI-22530	University of Minnesota Yunis, Edmond	150	3	1	$ 94,229 Renewal
AI-22531	Univ. of Texas, Dallas Stastny, Peter	45	2	1	$ 37,801 Renewal
AI-22536	Cleveland Clinic Braun, William	30	1	1	$ 12,663 Renewal
AI-22539	St. Un. of N.Y., Rochester May, Allyn	20	1	1	$ 11,225 Renewal
AI-42507	University of Utah Dewitt, Charles	20	2	1	$ 19,882 Renewal
AI-42551	St. Un. of N.Y., Brooklyn Koontz, Samuel	140	1	1	-0-(prior year) Renewal
AI-42552	Peter Bent Brigham Hospital Carpenter, Charles	100	3	2	-0-(prior year) Renewal
AI-52504	Univ. of Texas, Galveston Beathard, Gerald	10	1	1	$ 14,512 Renewal

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Evaluation of Tissue Matching in Renal and Bone Marrow Transplantation   Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings			FY'75 Obligations FY'76 Plans
		# of Transplants	# of Hospitals	# of Laboratories	
AI-52505	Univ. of Texas, San Antonio Dougherty, Joseph	30	2	2	$ 20,507 Renewal
AI-52506	Baylor College of Medicine Suki, W.	35	3	1	$ 37,620 Renewal
AI-52510	Albany Medical College Lempert, Neil	25	2	1	$ 17,152 Renewal
AI-52511	University of Arkansas Flanigan, William	35	2	2	$ 21,144 Renewal
AI-52512	University of Oklahoma Oleinick, Samuel	15	3	1	$ 10,096 Renewal
AI-52513	St. Univ. of N.Y., Syracuse Henry, John	15	1	1	$ 15,124 Renewal
AI-52514	St. Univ. of N.Y., Buffalo Kano, K.	20	2	1	$ 10,550 Renewal

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Antilymphocyte Globulin Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY'75 Obligations FY'76 Plans
AI-10001-05	National Naval Medical Center Sell, K.	Clinical Evaluation of the Efficacy of Rabbit Antihuman Thymus Globulin. (RAHT). Collects and evaluates clinical and laboratory data on patients treated with antithymocyte globulin. The Naval Medical Research Unit has obtained a FDA-IND to study this product developed and produced by them with joint NIAID/NMRI funding. The material of demonstrated efficacy in the prolongation of skin and renal graft survival in subhuman primates is being used in a double blinded clinical trial at the University of California at San Francisco. This Interagency Agreement provides funds for the collection and analysis of data on the clinical trial. All 100 patients have been entered on the study which is now in a two year follow up phase to determine late toxicity and efficacy.	$123,900 Renewal

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Antilymphocyte Globulin   Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY'75 Obligations FY'76 Plans
AI-22540	University of California Salvatierra, O.	Evaluation of RAHTG as an immunosuppressive agent. To collect and record clinical and laboratory information on patients on an RAHTG/placebo study, and to conduct special in vitro tests. This contract provides funds to collect laboratory and clinical data on the 100 patients in this double blinded, randomized study that is designed to determine if a program developed, RAHTG which is effective in subhuman primates, produces any significant improvement in the outcome of human cadaver kidney transplants. Under a Navy IND and protocols, the contractor has administered coded medications to patients. The Contract provides for funds for reporting the results of this study and for special laboratory tests to determine the patients immunologic status during the study. All 100 patients have been entered into the study at this time and a two year follow up period in underway, during which time clinical and laboratory findings will be sent to the Navy Statistical Center. Two years after the last patient was entered the code will be broken and the results analyzed. The information obtained will show the role that this preparation played in preventing allograft rejection.	$ 49,247 Renewal

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Regulation of Immune Responsiveness Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY'75 Obligations FY'76 Plans
AI-42531	The University of Texas, Dallas Health Science Center Beer, Alan E.	A study of the mammalian uterus as an apparent immunologic priviledged site. The contractor is to determine the cellular and humoral factors which control the uterus as an immunologically priviledged site. Thus far, the contractor has been able to demonstrate specific prolongation of skin grafts and transplantation of ectoplacental cones beneath the renal capsule of multiparous female rats. Since this activity is specific, they are attempting to isolate and identify a serum component responsible for this in vivo phenomena. In a chimeric in vivo model of Fischer female rats repopulated with Lewis spleen and/or bone marrow cells, this contractor has shown that (1) the Lewis constituent of the chimera can be suppressed, (2) such potentially active cells can cross the placenta, and (3) these cells are released from suppression postpartum. Apparently, serum factors are likely candidates for this immunological suppression and these can be further analyzed in GVH reactions reduction and capacity to prolong the lives of the offspring. These studies on in vivo models of the rat will be easiest to analyze for their specificity of reactivity.	$86,957 Renewal

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Regulation of Immune Responsiveness   Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY'75 Obligations FY'76 Plans
AI-42529	Baylor College of Medicine Rich, Robert R.	A study of cellular mechanisms whereby activated T cells regulate the response of normal T cells to alloantigens. The contractor has determined that regulator T cells may either help or suppress the reactivity of responder cells in MLC. He has also shown that regulator cells can be activated by exposure to Con A or to alloantigens and that some T cells may modulate alloagressive reactions. Recent evidence indicates that cells which mediate the helper function in MLC reactions have different homing properties in vivo than those which suppress and suggests that these two functions are mediated by different sub-sets of T cells. Hopefully, these two cell types can be isolated and purified by receptor avidity for Con A or other techniques. This contractor also has some preliminary evidence that suppressor T cells in vitro release suppressive factors into the media. This activity is reasonably stable at freezer temperatures and enhances the possibility of purifying and characterizing the suppressor activity. Establishment of the CML assay with human and mouse cells opens the area of determining whether the population of activated suppressor T cells which regulate the proliferative T cell response in MLC also regulates directly or indirectly the development of killer cells.	$89,300 Renewal

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Regulation of Immune Responsiveness   Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY'75 Obligations FY'76 Plans
AI-42533	Research Foundation of St. Univ. of New York Miller, Frederick	Study the immunosuppressive effect of alpha-globulin fractions in an effort to selectively suppress the immune response by altering antigen recognition. Obtain these fractions in pure form from the most effective sources and determine by in vivo and in vitro assays the mode of suppressor activity. Thus far, fifty potential immunosuppressive types of human and animal material have been screened for activity and four extraction procedures have been employed for isolation of this activity. Five active immunosuppressive substances were found by in vitro MLC, induction of plaque forming cells, and by in vivo cells mediated immunity tests. These sources are: 1) low molecular weight material from human serum, 2) high molecular weight material from human serum, 3) bovine serum, 4) human placenta, and 5) human spleen. Approximately 90% pure produce from low molecular weight human serum fraction has been obtained and it suppresses mice immunological mechanism and an MLC reaction. These and other methods of purification are being applied to human placenta and spleen factors. Dose response curves for the various materials on MLC have been established and immunologic reconstruction experiments will be used to examine their modes of action.	$85,479 Renewal

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Immunopathology   Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY'75 Obligations FY'76 Plans
AI-42505	Scripps Clinc and Research Foundation Wilson, C.	Study of the etiologic events and antigen-antibody systems involved in human glomerulonephritis and the course of nephritis in transplanted patients. To provide an immunopathological analysis of human kidney diseases by assessing serum and renal tissue before, during, and after transplantation. This service is provided to all U.S. Kidney Transplant Centers. Currently 76 such centers have participated in this service and significant contributions concerning the etiology and treatment of renal failure and graft rejection have been obtained.	$176,700 Renewal

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Publications and Conferences   Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY'75 Obligations FY'76 Plans
75-C-394	Sixth International Histocompatibility Workshop Conference Kissmeyer-Nielsen, F.	To plan for and conduct a Histocompatibility Workshop Conference held at Aahus, Denmark June 29 - July 3, 1975. This contractor provided facilities, and personnel for the packaging and distribution of human antisera and cells selected from worldwide sources to the laboratories participating in the workshop. A system was designed for the collection of data obtained in the study of histocompatibility antigens in a variety of races and disease states. This data was collected and analyzed to further define the various antigens involved in histocompatibility identification. A workshop was organized and conducted to discuss and evaluate the above activities, and the contractor will prepare, edit and submit to the printers a report of the results and conclusions of the conference. This report will be published as "Histocompatibility Testing 1975."	$12,500 None

INDIVIDUAL CONTRACT AND AGREEMENTS TABULATION

Program: Publications and Conferences   Branch: TIB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY'75 Obligations FY'76 Plans
75-C-447	American Association of Clinical Histocompatibility Testing Shaw, J.F.	Provide administrative, logistics and travel support for the planning and conduct of the Histocompatibility Testing Meeting held April 29 to May 1, 1975 in Birmingham, Alabama. The contractor prepared and distributed preliminary meeting announcements, developed a scientific program, and made the necessary arrangements for the Meeting. Travel subsidies were provided for selected speakers, an abstract booklet was prepared and distributed and the contractor will provide the NIH with a scientific summary report of the meeting.	$ 8,350 None

PUBLICATIONS

A - CONTRACTOR PUBLICATIONS

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Braun, W.E., Banowsky, L.H., Nakamoto, S., Straffon, R.A., and Magalhaes, R.: Lymphoceles associated with renal transplantation. Proc. Dialysis and Transplant Forum, Nov. 1974.

Braun, W.E., Banowsky, L.H., Straffon, R.A., Nakamoto, S., Kiser, W.S., Popowniak, K.L., Hewitt, C.B., Stewart, B.H., Zelch, J.V., Magalhaes, R.L., Lachance, J.G., and Manning, R.F.: Lymphoceles associated with renal transplantation: Report of 15 cases and review of the literature. Am. J. Med. In press.

Braun, W.E., Crile, G., Jr., Straffon, R.A., Nakamoto, S., Isbister, W.H., Gifford, R., Deodhar, S., Khanna, R., and Banowsky, L.H.: Thymectomy and splenectomy in long-term (6 to 11 year) renal allograft recipients. Transplant. Proc. In press.

Buckner, C.D., Clift, R.A., Fefer, A., Lerner, K.G., Neiman, P.E., Storb, R. and Thomas, E.D.: Marrow transplantation for the treatment of acute leukemia using HL-A identical siblings. Transplant. Proc. In press.

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Fefer, A., Mickelson, E. and Thomas, E.D.: Leukemic antigens: Mixed leukocyte culture tests on 12 leukemic patients with identical twins. Clin. Exp. Immunol. In press.

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Grecek, D.R., and Braun, W.E.: Escape from sensitization of normal and leukemic lymphocytes. Transplantation 18:187, 1974.

Kroener, C.A., Perry, V.P., Martin, J.L., Sasso, J.C.: Freeze drying of histocompatibility typing sera. Cryobiology. In press .

Magalhaes, R.L., Braun, W.E., Straffon, R.A., Hoffman, G.C., Shainoff, J.R., Osborne, G., and Deodhar, S.D.: Microangiopathic hemolytic anemia (MAHA) in renal allotransplantation. Am. J. Med. In press.

McHenry, M.C., Alfidi, R.J., Deodhar, S.D., Braun, W.E., and Popowniak, K.L.: Hospital-acquired pneumonia. Med. Clin. North Am. 58:565, 1974.

Miller, F.: Serum Derived Immunosuppressive Substances I. Partial Purification and Range of Action. Transplantation. In press.

Neiman, P., Thomas, E.D., Buckner, C.D., Storb, R., Fefer, A., Glucksberg, H. Clift, R.A., and Lerner, K.G.: Marrow transplantation for aplastic anemia and acute leukemia. Ann. Rev. Med. 25: 179-198, 1974.

Perry, V.P., Martin, J.L. and Kroener, C.A.: Nutrient Medium and Frozen Storage of Human Lymphocytes. Cryobiology. In press.

Rich, Solliday, S., Rich, R.R.: Regulatory mechanisms in cell-mediated immune responses. I. Regulation of mixed lymphocyte reactions by alloantigen-activated thymus derived lymphocytes. J. Exp. Med. 140: 1588-1603, 1974.

Rich, Rich, R.R., Solliday, S.: Biological expressions of lymphocyte activation. IV. Concanavalin A-activated suppressor cells in mouse mixed lymphocyte reactions. J. Immunol. 114: 1112-1115, 1975.

Rodriguez, B.A., Rich, R.R., Rossen, R.D.: Determinants of differential migration of mouse spleen cells treated with concanavalin A. In press.

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Storb, R., Thomas, E.D., Buckner, C., Clift, R.A., Fefer, A., Glucksberg, H. and Neiman, P.E.: Transplantation of bone marrow in refractory marrow failure and neoplastic diseases. Am. J. Clin. Pathol. In press.

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Thomas, E.D., Storb, R., Clift, R.A., Fefer, A., Johnson, F.L., Neiman, P.E., Lerner, K.G., Glucksberg, H. and Buckner, C.D.: Medical Progress: Bone Marrow Transplantation. N. Engl. J. Med. April 17, 1975, 832-843.

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B - STAFF PUBLICATIONS

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Ray, J.G., Jr., Hare, D.B., Pedersen, P.D., and Kayhoe, D.E., (Eds): Manual of Tissue Typing Techniques, September 1974. U.S. Dept. of Health, Education, and Welfare, National Institutes of Health, Bethesda, MD, 1974, 173 pp.

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INFECTIOUS DISEASE BRANCH

The Infectious Disease Branch (IDB) supports research efforts which are designed to develop materials and methods which will significantly improve our ability to control major infectious diseases in the United States. To a large extent, these efforts result in the application of basic research findings to clinical medicine. While a majority of projects supported by the IDB involve the utilization of currently available research tools and techniques, support is also provided for a limited number of basic studies needed to fill critical scientific gaps in existing programs. The IDB contracts involve research efforts in five program areas: Venereal Diseases, Hepatitis, Influenza and other Viral Respiratory Infections, Antiviral Substances and Disease Control Measures.

I. Venereal Diseases - Venereal Diseases Contracts

Venereal disease is the major infectious disease public health problem in the United States. At a workshop of experts in this field, sponsored by the IDB in July, 1973, a number of research areas appropriate for the contract support mechanism were identified. Included in those areas were studies on the cultivation of *Treponema pallidum*, the causal organism of syphilis, and the development of a serologic typing system for *Neisseria gonorrhoeae*.

Three contracts were awarded during FY 74 for studies directed toward *in vitro* cultivation and propagation of the syphilis spirochete, with emphasis on the use of modern tissue culture techniques. These contracts have supported some promising work toward the development of improved media for maintenance of viable *Treponema* and toward finding a tissue culture system which may prove of value in propagating this organism.

Plans are now underway for initiating two or three contracts to develop a serologic typing system for the gonococcus. It is hoped that a better understanding of the organism's antigenic structure and a practical serologic typing system will result from the studies. Not only would such a typing system be valuable in epidemiological studies of gonorrhea and assist in the diagnosis of asyptomatic infection, but also it would provide information essential for developing gonococcal vaccines.

II. Hepatitis Program - Hepatitis Contracts

The goal of the hepatitis program is to support research activities leading to the control of hepatitis A and B infections. Major emphasis is being placed on studies of hepatitis B virus (HBV) infection since recent knowledge of HBV surface and core antigens (HB_SAg, HB_CAg) and antibodies (anti-HB_S, anti-HB_C) characteristics have made it possible to better define this disease. The projects supported resulted from careful Institute planning, which included a series of workshops. Although major breakthroughs have occurred in the identification of the causative agent of hepatitis A, progress in this area has been severely hampered by the unavailability of *Saguinus mystax* marmosets. Collaborative agreements with the Center for Disease Control and Bureau of Biologics, FDA, have been undertaken to obtain these animals which have been shown to be the most suitable model for the study of hepatitis A pathogenesis and host serologic response. The studies presented in this report represent collaborative efforts of our intramural scientists, NIAID contractors, and other cooperating government agencies.

1. VACCINE DEVELOPMENT AND TESTING

Development of an inactivated vaccine consisting of purified 22nm surface antigen (HB_sAg) received considerable impetus this past year. A prototype inactivated vaccine of subtype ayw was prepared from a unit of blood of known infectivity and antigen content. The vaccine was tested in chimpanzees for safety, antigenicity, and protection against challenge. None of the vaccinated animals developed evidence of HBV infection as judged by lack of rise of SGPT activity, development of HB_sAg, or development of anti-HB_c. The vaccinated chimpanzees developed anti-HB_s as measured by sensitive serologic assays. The antibody titers reached a peak within approximately one month after a booster inoculation and diminished in titer by the end of six months follow-up.

At the end of six months, protection was evaluated by challenging the vaccinated animals with known infectious doses of HBV, subtype ayw. Control animals developed hepatitis B as judged by a rise in SGPT activity, development of persistent HB_sAg, and the development of anti-HB_c. In contrast none of the vaccinated animals had any biochemical or serologic evidence of HBV infection. Thus, immunization with purified HB_sAg appears to stimulate protection against HBV infection.

Efforts to produce and evaluate such a vaccine for use in man are now underway. The source material, human plasma containing HB_sAg from chronic carriers, is being procured through two contracts. One hundred liters of subtype adw have been obtained and are being pooled, safety tested, and purified for vaccine preparation. Similar preparation of a vaccine from subtype ayw should begin in the near future.

Before use in man is contemplated, the inactivated vaccines will be extensively tested for safety, antigenicity, and efficacy in expanded numbers of chimpanzees. The numbers of seronegative chimpanzees required for these experiments will virtually deplete the stock of susceptible animals that have been obtained through contracts. The NIAID currently supports three contracts for supplying colony born animals, research facilities, and holding areas. The IDB is investigating the feasibility of supporting another chimpanzee colony for future vaccine work.

Although plans for immunizing humans are at the most preliminary stage, it appears that the first volunteers should be individuals not "at risk" for hepatitis B. Intensive follow-up of the first vaccinees will establish the safety and antigenicity of the vaccines. Only after conclusive evidence of vaccine safety has been established, will the question of efficacy be investigated. These studies will by necessity be performed in as yet-to-be-identified "at risk" populations. It is anticipated that the test populations will be identified and studies supported by the contract mechanism. Plans for volunteer testing and evaluation will be developed during the forthcoming year.

2. PASSIVE IMMUNIZATION

In FY 73 the NIAID, in collaboration with the Veterans Administration (VA) hospitals, initiated studies to determine if high-titered anti-HB_S gamma globulin can prevent or modify HBV infection in persons experiencing accidental parenteral exposure (Needlestick) or in persons receiving blood transfusions. While normal gamma globulin preparations have little or no effect in preventing icteric hepatitis B infection, preliminary evidence available suggests that gamma globulin containing anti-HB_S may be effective in preventing this disease. Since these are randomized, double-blind studies, no results are available at the present time. The studies are monitored and reviewed periodically by the Operations Committee of the VA Hepatitis Cooperative Studies. After entry of the last subject into the studies, an additional year of follow-up and data compilation will be required. The gamma globulin preparations for these studies were procured by the NIAID from Cutter Laboratories of Berkeley, California.

3. EPIDEMIOLOGY

In order to help identify "high risk" populations for further passive or active immunization trials, the IDB is supporting a Hepatitis Study Center at the West Seneca State School, Buffalo, New York. In addition to evaluating "at risk" subjects, this study is providing information on incidence, mode of transmission, and dynamics of hepatitis B in an institutionalized population.

The data accumulated thus far indicate that the attack rate for hepatitis B infection in the resident population is high. HBV infection is associated with evidence of liver damage in over 20% of antigen positive individuals although overt clinical illness is not seen. Only 20% of new infections in the resident population were associated with demonstrable HB_SAg, but this is probably an under-estimation, since the population was bled only twice during the one year observation period.

The high HBV attack rate at West Seneca State School yielding 150-200 new HBV infections per year, indicates that a candidate vaccine could be tested for efficacy in this population. Seroconversion to anti-HB_S from natural infection as distinct from the vaccine could be differentiated by the RIP assay for anti-HB_C, characteristic of HBV infection only. This presupposes that the vaccine has been tested for safety and antigenicity in a not-at-risk population and has been found absolutely acceptable.

In considering a vaccine efficacy trial in a mentally retarded population that includes children, care must be given to the benefit/risk ratio. The finding of a high HBV attack rate in this population and its association, in a proportion of individuals, with biochemical evidence of liver damage indicates that the population would benefit from a hepatitis B vaccine. Hypothetical negative consequences of such a vaccine would be weighed against positive identifiable factors prior to any vaccine evaluation.

Another population where the vaccine could be evaluated for efficacy is the staff in hemodialysis centers. Preliminary evidence exists that there is a high incidence of clinical illness in this group. Efforts should be initiated in the near future to ascertain if this population would represent an appropriate "at risk" group for illness from hepatitis B and to establish baseline incidence data to determine if the numbers are sufficiently large to perform an efficacy study.

4. LABORATORY SUPPORT

The NIAID currently supports the Hepatitis Testing Laboratory at the American Red Cross. The laboratory's function is to test for hepatitis antigens and antibodies by the most sensitive techniques available. Tests available include subtype identification, radioimmunoassay procedures for antigen and antibody, and passive hemagglutination tests. During the past year, the laboratory has performed hepatitis B antigen and antibody tests for the NIAID hepatitis programs, and also for the hepatitis programs of other NIH Institutes and the Food and Drug Administration. An additional function of the laboratory is to aid in the standardization of methods for detecting hepatitis antigens and antibodies.

Currently one of the most sensitive techniques for the detection of HBV antigens in tissue consists of immunohistochemical assay procedures. In FY 73 the NIAID established a contract for applying immunohistochemical assay techniques to the detection of hepatitis B antigens, particularly in primate specimens from animals involved in hepatitis infections. Results from these studies have contributed greatly to the concept that the DANE particle is indeed the virion of hepatitis B. By using highly specific reagents, direct and indirect immunofluorescent techniques, Dr. Edgington, Scripps Clinic and Research Foundation, has shown that the core particle is produced in the nucleus of the hepatocytes and that the coat antigens are synthesized in the cytoplasm. The complete DANE particle is found in the hepatocyte nucleus, cytoplasm and plasma. During the next two years, there will be a continued requirement for pathological and immunohistochemical assays of tissues from animals receiving the candidate hepatitis B vaccine.

III. Influenza and Other Viral Respiratory Infections

The goal of the IDB's Viral Vaccine Program is to develop vaccines for prevention of serious infectious diseases for which adequate prophylactic and/or therapeutic measures are not now available. Vaccine development studies are being performed by the scientists in the Laboratory of Infectious Diseases (LID), and under contractural arrangements. Scientists at LID are preparing mutants to influenza viruses, and respiratory syncytial virus (RSV) for use as vaccines, while Dr. Simpson at Rutgers University is developing live virus mutants to RSV and parainfluenza virus types 1, 2 and 3. Virus vaccines and virus challenge pools are prepared and safety tested by Flow Laboratories.

A contract was initiated to search for evidence that all or a portion of the genome of influenza virus, RSV, or parainfluenza type 3 virus persists in the cells of the respiratory tract of man either in the form of viral RNA or as integrated complementary DNA. If any of these viruses is found to persist naturally in the respiratory tract of man, the safety of live virus vaccines will have to be re-examined.

The IDB has emphasized two kinds of vaccine evaluation centers: general study centers where the principal investigator has interest and expertise in the broad area of vaccine evaluation, and special study centers where the principal investigator has a primary interest in evaluating a particular type of vaccine. Included in our general study centers are the University of North Carolina, D. C. Children's Hospital, Baylor College of Medicine, Vanderbilt University, University of Colorado and the University of Rochester. Each of these contracts is performing studies under at least two of the vaccine program areas.

A. Influenza Program - Contracts

Establishment of a formal program on influenza within the Infectious Disease Branch of Collaborative Research began in FY 75. Acting within the framework of recommendations from the Influenza Steering Group and the Infectious Disease Committee, the IDB began expanding research efforts on the viral disease frequently referred to as the "last plague of mankind". The long range goal of the Infectious Disease Branch Influenza Program is to support research activities leading to the knowledge that will abort or lessen impacts of future epidemics and pandemics. Areas of IDB research support are closely coordinated with other agencies of the Government (particularly BoB, CDC, and Army) in order to avoid overlap or duplication of efforts. In addition, much of the current IDB program is directly related to the efforts of the intramural program in the development and evaluation of an attenuated vaccine.

1. VACCINE RESEARCH AND DEVELOPMENT

During the past year, studies have been conducted with live attenuated (ts) vaccines, neuraminidase-specific inactivated vaccine, and a developmental subunit vaccine.

Further testing of the live Hong Kong ts-1-E and another recombinant, Udorn ts-1-E, was performed in normal adults, and children seronegative for hemagglutinin and neuraminidase antibodies. These studies were conducted at vaccine evaluation centers at the University of Rochester, University of Virginia, D. C. Children's Hospital and Vanderbilt University. As reported last year, the Hong Kong ts-1-E recombinant has been shown to be safe and immunogenic in normal and elderly adults. However, vaccine administration to seronegative children resulted in reversion of the ts phenotype. Although the ts virus reverted, there was no conclusive evidence of illness resulting from the vaccine.

Recombinants of the H_3N_2 Udorn virus containing the ts-1-E lesions have also been satisfactorily tested in normal adults, and trials were initiated in children (ages 2-5 years).

The instability of the ts-1-E marker in the candidate vaccine has been a temporary setback in the NIAID efforts to produce a live, attenuated influenza vaccine. Ideally, such a vaccine would be genetically stable, infective, and immunogenic. Efforts are presently being directed towards evaluation of another recombinant, Hong Kong ts-1-A previously thought to be overattenuated for man.

In order to learn the nature of the ts lesions of the various attenuated influenza A mutants and recombinants, Dr. Robert Simpson, Rutgers University, has a contract to investigate and define the genetic and biochemical defects of the viruses. In addition to sponsoring the study on genetic analysis of the viruses, Dr. Steven Mostow, Case Western Reserve University, is investigating the development of an in vitro assay for degrees of attenuation for influenza viruses. There is a lack of information on the nature of genetic defects producing attenuation, and the transfer of the ts characteristic to other influenza variants produces recombinants of varying degrees of attenuation. Proper levels of attenuation can only be determined in volunteers at the present time. Dr. Mostow has observed that there is considerable correlation for ciliary activity of tracheal organ cultures with degrees of virulence of mutagenized or recombinant influenza viruses. If this in vitro system can be further defined, the assay will be invaluable for selection of future live vaccine recombinants as antigenic drift or shift dictate production of recombinants with different surface antigens.

An experimental inactivated influenza vaccine (monospecific for the neuraminidase surface antigen) is currently being evaluated for safety, antigenicity, and, hopefully, efficacy in a small field trial in Buffalo, New York. Dr. Pearay Ogra, Children's Hospital, Buffalo, has vaccinated 400 school children (ages 9-14) with the inactivated neuraminidase vaccine. Controls include 400 children vaccinated with monovalent H_3N_2 inactivated vaccine, and 200 children receiving a saline placebo. All vaccinees accepted the vaccines without untoward reactions. There was an outbreak of influenza A this past winter, so efficacy results should be available later this year.

Pediatric populations are not considered "high risk" populations for influenza, but morbidity among school children is very high. To this end, it would be desirable to break the transmission chain of influenza by inducing immunity in this population. The current licensed inactivated influenza vaccines are not recommended for children because of systemic reactogenicity. There is, however, evidence that the various components of the influenza virus retain antigenicity when properly isolated. Dr. Robert Webster, St. Jude Children's Hospital, is currently investigating the feasibility of producing a protein subunit vaccine for pediatric populations. A preliminary preparation was inoculated into adult volunteers last year, and serologic evidence indicated that the subunit preparation was as antigenic as the whole virus vaccine. However, further purification of the components of the subunit vaccine is required, and it will require several years before the evaluation of this approach can be undertaken.

2. IMMUNOLOGY

Various aspects of the immunological response to live and inactivated influenza vaccines are being conducted at the Baylor College of Medicine (Influenza Research Center) and the University of Rochester. There is a paucity of definitive data on protective mechanisms from influenza infection, and correlates of immunity. The Influenza Research Center has described lymphocyte-mediated cytotoxicity (LMC) to influenza virus in vitro. The assay is a modification of the LMC test system used for rubella and measles and is reproducible, specific and sensitive.

3. EPIDEMIOLOGY

There are numerous questions on the epidemiology of influenza that remain unanswered. The Influenza Research Center at the Baylor College of Medicine has started a variety of surveillance studies designed to detect the earliest signal of influenza activity in a community, methods for detection of epidemics, and longitudinal family surveys. The Houston area experienced a moderate outbreak of influenza this past winter, and the data accumulated are being compiled and analyzed. Since this facility was just established last summer, epidemiologic programs are being initiated and evaluated, so that significant results should be available in future years.

During the past several years, the knowledge of influenza virus infections among animals has expanded at a rapid rate. The major implications of animal infections for man is clear. This is concerned with the hypothesis that major antigenic shifts, characteristic of new human pandemic strains, have resulted from the recombination of influenza viruses in some animal species. The NIAID is in the process of awarding a contract to Dr. Robert Webster, St. Jude Children's Hospital to conduct intensive epidemiological and epizootiological studies, combined with antigenic and biochemical analyses of influenza viruses in order to ascertain the significance of animals in the evolution of new human strains. This contract will also provide a repository for all newly isolated and characterized animal viruses and appropriate reference reagents. These studies will be coordinated with existing World Health Organization activities to ensure a complete international surveillance network.

4. PATHOGENESIS

Uncomplicated influenza has been shown to produce pathologic alterations in the tracheobronchial tree and abnormalities in lung function in the absence of demonstrable changes on chest X-rays. Moreover, bacterial pneumonia is the most common and significant complication of influenza. Defense of the lower respiratory tract and lung against microorganisms is therefore of importance. The nature of this defense and the relative significance of different mechanisms in man are presently unknown. The Influenza Research Center will be studying the effect of naturally occurring influenza on the development of humoral and cellular defense mechanisms in the normal tracheobronchial tree and lung, and assessing measurable alterations in pulmonary function during the course of naturally occurring influenza. These studies will be initiated during the next contract year of the Influenza Research Center.

B. Parainfluenza Viruses - Contracts

Parainfluenza virus type 3 is one of the principal causes of severe lower respiratory tract disease among infants and children. The need for an effective vaccine to reduce the morbidity and economic toll of this pediatric viral illness remains apparent. Fourteen ts mutants of type 3 parainfluenza have been isolated in primary BEK cell cultures from chemically mutagenized stocks of virus. Additional studies on these mutants will continue in order to determine which of them are attenuated and immunogenic for hamsters and also satisfy criteria for both genetic stability and lack of persistence in mammalian cells. Mutants to type 1 and 2 parainfluenza viruses will be derived.

C. Respiratory Syncytial Virus - Contracts

Since RSV is one of the principal causes of severe lower respiratory tract disease among infants and children, there is need for an effective vaccine. RSV has also been implicated in asthmatic attacks, and in apnea in infants living at high altitudes. Scientists from LID, and from Rutgers University have obtained RSV mutants which appear genetically stable, and, in animal studies, to possess the appropriate degree of attenuation. Viral vaccines can be prepared and evaluated in humans. However, during this contract year, the contractor at Rutgers has made the very significant discovery that RSV can enter into association with host cells in the form of a proviral DNA intermediate. This finding is being pursued further in order to understand its full significance in relation to the disease process, to discover whether the phenomenon occurs with other candidate viruses, and to determine whether the capacity to form viral DNA intermediates should obligatorily preclude the use of live attenuated strains of human RNA respiratory viruses as vaccines. A workshop on viral persistence is being planned.

The ferret and the calf have been selected as experimental animal models which develop disease similar to that seen in young infants hospitalized with RSV bronchiolitis or pneumonia. Studies will be performed to determine the role of immunologic factors in resistance to RSV infection and disease as well as their possible participation in the immunopathologic potentiation of disease. Two contracts for these studies will be awarded during the current fiscal year.

D. Adenoviruses - Contracts

Crystalline adenovirus type 5 hexon and fiber antigen vaccines were prepared by Dr. Julius Kasel, Baylor College of Medicine. Both vaccines have been shown to be antigenic and safe in adults, and young teenagers. Dr. Kasel is preparing hexon and fiber antigen vaccines from type 4 adenovirus. The type 4 subunit vaccines will be tested in adults in order to ascertain if they are primary immunogens.

A contract was initiated with St. Louis University to study both normal tissues and tumors for the presence of DNA sequences specific for adenoviruses 2, 7, and 12 and to determine if viral DNA sequences in tumor cells represent infectious virus or integrated viral genes. This contract is funded through the BoB, FDA, with a portion contributed by the IDB because of our past and possible future interest in whole adenovirus vaccines.

IV. Antiviral Substances Program - Antiviral Contracts

There are many viral diseases for which there is no prophylaxis or therapy presently available and which constitute serious public health problems. There is, therefore, a need for the development of antiviral substances for the treatment of these diseases. Planning for a program to help meet this need began in July, 1969. At that time a thorough study of the field revealed that there was considerable and increasing interest in this area and that interferon or interferon inducers were the most promising antivirals to exploit. This continues to be the case; in fact, there is increased interest in interferon as a result of studies which indicate that it may be effective against osteogenic sarcoma. There is also considerable and growing interest in chemotherapeutic agents such as adenine arabinoside (ara-A), cytosine arabinoside (ara-C), and disodium phosphonoacetate (PAA). The program continues to be alert to new developments emanating from scientist-originated basic research in order to provide the needed stimulus to bring this knowledge into practical use in man. The intent of the program is to serve as a focus for all activities on antivirals conducted by Extramural, Intramural and Collaborative Research of the National Institute of Allergy and Infectious Diseases and to some extent, other Institutes of the National Institutes of Health.

Following the advice of advisory groups including the Infectious Disease Committee, there has been a gradual shift of emphasis toward more clinical studies, as the original goals in basic studies to fill some of the gap areas are accomplished. There are also increased efforts with antivirals other than interferon such as studies on ara-A and the initiation of studies on PAA.

1. NONCLINICAL RESEARCH AND DEVELOPMENT

Standards and Reagents: In collaboration with the Research Resources Branch, the ASP has successfully established reference reagents for rabbit interferon, mouse interferon, and interferon inducers [polyinosinic·polycytidylic acid (poly I·poly C)]. The two interferon preparations (rabbit and mouse) were originally prepared in the wet frozen state. The ASP has now made available for experimental use lyophilized preparations of mouse interferon and also human interferon. These reagents have undergone standardization analyses with unitage assigned which properly corresponds to the original materials established as the standards. A study is underway to prepare a lyophilized rabbit interferon standard, since this is the one remaining wet frozen interferon reagent. This procedure requires some innovative techniques since all interferons have proven difficult materials to work with in respect to their stability and resistance to manipulation. A contract for the production of specific antisera against human interferon by conventional methods was completed and terminated. This product does have some non-specific protein material; therefore, a new contract was initiated utilizing newly developed techniques to obtain monospecific antibody. This will employ mutagenized cells, cloning and tissue culture techniques. The previous material is being used to more accurately determine the nature of interferon and for affinity chromatography in interferon purification. Utilizing this antisera, scientists were able to show that there are at least two types of interferon with different properties. It is hoped that a more potent antiviral can be developed. This new approach to the production of antisera will hopefully yield a more useful reagent for these and other studies.

Purification: If exogenous interferon is to be used in man, there is a need for purification and concentration. The ultimate goal would be purification to the extent that the chemical structure can be defined and eventually interferon manufactured synthetically. Currently studies on purification of human interferon are being conducted through a collaborative effort involving NIAMDD and both intramural and collaborative components of NIAID. The use of affinity chromatography techniques utilizing specific interferon antisera has proven very useful in these studies. As progress is made with purification, it becomes more important to develop techniques for stabilization of this material. Human diploid cell interferon is less stable than leucocyte interferon and partially purified interferon less stable than crude. A contract is underway to determine the most effective ways of maintaining the stability of this material. The contractor has spent the initial year developing the baseline data and testing the routine stabilizing procedures and is currently initiating studies on additives, gaseous atmospheres, etc.

Production - Exogenous and Endogenous: If interferon proves effective in the treatment of human viral diseases, two methods of administration are available: the administration of exogenous interferon, produced *in vitro* or endogenous interferon through the use of inducers. For the former, there is a need for the development of techniques which will provide a more efficient, safe and economically feasible method of producing large quantities of human interferon. A wide variety of cells have been tested to determine their relative capacity to produce interferon using a series of inducers. There is very little difference between cells in their ability to produce interferon, but human amnion cells and foreskin fibroblast diploid cells are the most promising. Two contracts are supported in this area, one to develop better production methodologies, including superinduction (NYU). This contract continues to be very productive. They also provide crude human diploid cell interferon for purification studies. A new contract was awarded this year to develop hardware for scaling up production processes developed by NYU. Success of this program is evidenced by the interest expressed by potential commercial producers. A number of the latter have requested and received the most productive cell developed by the NYU contract. The program is also collaborating with intramural staff on the development of a new and promising interferon inducer (poly I·poly C-lysine complex). The appropriate animal studies have been done and evaluation in humans is about to begin. Two contracts to determine which of the biological interferon inducers provides the greatest potential for clinical potency are in effect. Materials developed are currently being evaluated by the Cooperative Antiviral Testing Group.

Animal Models: As part of the effort to develop potential antivirals for clinical use, a series of animal-viral disease models, which closely mimic diseases in man, was established. This constitutes the Cooperative Antiviral Testing Group of the ASP. The models selected encompass as wide a range of animal species and viruses as possible with the exception of the nonhuman primate. Utilizing these models, comparative *in vivo* evaluation of various antivirals which show some promise as prophylactic and/or therapeutic agents is possible. The mode and dose of infection have been selected to approximate the natural mode of infection as much as possible. Baseline data continue to be accumulated using interferon (exogenous and endogenous), and this will then serve as the reference for comparison of new antivirals as they develop. If any one of these compounds proves to be efficacious and non-toxic, human studies can be considered. Examples of this are demonstrated by the finding that ara-A and PAA have potential for clinical use. These compounds are currently being evaluated for use in man. During the past year, two new contracts were initiated to include a nonhuman primate model and the cat so as to broaden the animal species under study to include animals other than the standard laboratory animals.

2. CLINICAL EVALUATION

Clinical Studies: The state of the art has progressed to the point where some clinical trials are now underway and several others could be initiated. This is occasioned by better production methodologies for exogenous interferon; sufficient studies to show high levels of exogenous human interferon are not toxic, and also the development of a more effective inducer (poly I·poly C-lysine complex). A study to evaluate poly I·poly C against herpes keratoconjunctivitis has been completed and manuscript submitted for publication. This inducer was found to be as effective as the currently accepted drug, IDU, although neither drug provides optimal efficacy.

Exogenous human leucocyte interferon is being used in prophylactic studies for prevention of the recurrence of herpetic keratoconjunctivitis in patients who have a history of this disease. It has been shown that individuals who have had two episodes of herpetic keratoconjunctivitis have a 40% chance of having a recurrence within the next two-year period. Exogenous interferon is also being studied in the treatment of disseminated herpes zoster, which is a major problem in immunologically compromised patients. These studies were selected for support because it is anticipated that interferon will have its most beneficial effect in disease states that are dependent on de novo viral replication for their pathogenesis. Animal studies have indicated that interferon has a definite role in post-exposure treatment of rabies. The results are sufficiently encouraging to point toward eventual clinical evaluation.

Since exogenous interferon was shown to be efficacious against upper respiratory infections caused by rhinoviruses and influenza, a small effort has been initiated in this area. The early studies showed that huge quantities of interferon were needed to demonstrate an effect. This was clearly out of proportion to that expected from tissue culture and animal model extrapolation studies. Therefore, a contract is in effect to determine whether there is a mucocutaneous barrier which prevented the interferon, applied by drops, from reaching the affected cells. Initial studies are being done using biopsy material from humans and animal studies; findings from these systems will then be tested in man.

Preliminary studies in serious viral infections such as herpes encephalitis had suggested that IDU and ara-A may have definite beneficial results. A study at the University of Alabama was, therefore, developed to test the efficacy of these drugs. This contract has already been instrumental in withdrawing IDU from use against herpes encephalitis. Contrary to anecdotal publications, this double-blind study showed that the drug was not only not effective, but actually toxic to these patients. This portion of the study has been terminated. However, the ara-A study seems quite promising. A preliminary look at the data indicates that the drug is effective in some of the sequelae of varicella-zoster in immunosuppressed patients. However, greater numbers and a true double-blind study are needed. It is expected that, because of the apparent relatively low toxicity of ara-A, a study to evaluate its efficacy versus placebo versus PAA can be evaluated against cutaneous herpes.

V. Disease Control Measures - Research and Development

A. Acute Bacterial Diseases Contracts

1. MENINGITIS CONTRACTS

Two organisms (Hemophilis influenzae type b and Neisseria Meningiditis) are primarily responsible for bacterial meningitis in young children and infants. There is epidemiologic evidence that in children between the ages of 3 months and 6 years, the annual incidence of serious infections with H. influenzae is about 40 cases per 100,000 and of meningococcal C meningitis, 20 cases per 100,000. Infants 3 months through 2 years of age are at highest risk of serious infection. Effective immunoprophylaxis would be of great benefit to this segment of the pediatric population.

A candidate H. influenzae type b polyribose phosphate (PRP) vaccine has been developed by Drs. Porter Anderson and David Smith at the Children's Hospital Medical Center in Boston. This group, as well as the contractors at the University of Southern California, the University of Colorado, and the University of Rochester have been performing clinical evaluation studies of PRP in children. Children's Hospital, Boston, serves as a serological reference laboratory performing radioimmunoassays for anti-PRP antibodies in most sera obtained in these studies. The results of clinical studies to date indicate that PRP is immunogenic in children over the age of 16 to 24 months but that there is very little serological response in a substantial portion of infants under one year of age. The significance of this low level antibody response in infants with regard to protection is unknown but some investigators feel that even the low level may be protective. The H. influenzae PRP is being used as a control vaccine in a field trial of the meningococcal A polysaccharide vaccine in Finland (described below).

Dr. Anderson of Boston Children's Hospital has developed a new candidate H. influenzae vaccine which is prepared via a slight variation in the purification procedure. The resulting product consists of the PRP combined with a small amount of protein. The new candidate vaccine has been designated PRP-X and has, unlike the PRP, been shown to be highly immunogenic in weanling mice. It may prove to be more immunogenic than PRP in infants. The vaccine, PRP-X, has been tested for toxicity and antigenicity in a small number of adults. This work will be extended to a larger group of adults. If the adult studies appear promising, further clinical evaluation in infants will be carried out by the meningitis vaccine contractors.

Clinical studies in children of the groups A and C meningococcal polysaccharide vaccines developed by the Walter Reed Army Institute of Research are being carried out by contractors at the University of Connecticut, the University of Colorado, and the University of Southern California. The contract with Rockefeller University supports a reference testing laboratory to measure, via radioimmunoassay, the antibody response in sera from infants and children immunized with groups A and C meningococcal polysaccharide vaccines.

In general, results from clinical studies indicate that the quantitative ability of infants to produce antibodies to the meningococcal groups A and C polysaccharides is a function of age. Three month old infants respond weakly but consistently to the group C antigen and weakly and inconsistently to the group A antigen. Older infants (7-18 months) respond consistently to both antigens.

Results obtained by the University of Connecticut group indicate that injections of group C polysaccharide given at 12 months to infants who had received a primary injection at three months result in less antibody production when compared to a single injection given at the later time. The A vaccine behaves differently, and a booster effect can be demonstrated at one year in children receiving a primary dose at three months.

Because of the observation of the "dampening" of the group C vaccine when three months old infants receive the vaccine, no new clinical studies are at present being planned with the group C vaccine in this age group except for some small experiments designed to confirm the Connecticut results. The Branch is following with interest basic studies on the immunologic response of infants to group C vaccine and the recently reported studies on a new group C polysaccharide which is a variant of the C antigen currently under study.

During the past year, the IDB initiated a contract with the Central Public Health Laboratory, Helsinki, Finland, to conduct an efficacy trial of the group A meningococcal polysaccharide vaccine in children, ages 3 months to 6 years. The project resulted from a request for a study by representatives of the laboratory and the Finnish National Board of Health. Finland has, since 1973, been experiencing a moderate epidemic of group A meningococcal disease. The meningococcal vaccine was supplied by Merck, Sharp and Dohme and the control vaccine, Hemophilus influenzae PRP, was prepared under the IDB contract with Boston Children's Hospital. The H. influenzae PRP was selected as a control vaccine because of the high incidence of H. influenzae meningitis in Finland.

Approximately 50,000 children received the meningococcal group A vaccine and 50,000 the H. influenzae PRP in November, 1974. Boosters were given to children under 18 months of age in February, 1975. Both vaccines have been well tolerated by the children in the Finnish study. Results on disease incidence are too preliminary to determine efficacy of the vaccine at this time.

On the advice of our Infectious Disease Committee to explore the possibility of conducting a trial of meningitis vaccines sometime in the future in this country, an addition to the workscope of the University of Southern California has been made to allow for development of a meningitis surveillance system in Los Angeles County. This will be a pilot study to determine the feasibility of this type of surveillance and hopefully to provide a baseline of meningitis incidence in this large metropolitan area.

2. PNEUMOCOCCAL CONTRACTS

Monovalent and polyvalent vaccines of pneumococcal capsular polysaccharides have been developed and clinical evaluation studies conducted. Most of the work on vaccine development and the epidemiology of pneumococcal infections has been done through a contract with the University of Pennsylvania. A vaccine trial using two hexavalent vaccines was initiated in January, 1973, at Dorothea Dix Hospital, Raleigh, North Carolina. Immunization was completed in January, 1975, and a total of 1,300 volunteers received either two hexavalent pneumococcal polysaccharide vaccines or a saline placebo. Follow-up for disease incidence will be carried out through January, 1976. No serious adverse reactions have been observed. The vaccinees, a majority of whom are 50 years of age or older, have responded well to each of the 12 antigens. Results available to date indicate that the efficacy of the vaccine in preventing pneumococcal infection by types contained in the vaccine will be assured with statistically significant data.

A large scale trial is underway to determine the efficacy of a dodecavalent pneumococcal vaccine in preventing type-specific pneumococcal pneumonia and its impact on pneumococcal pneumonia and total respiratory disease in adults over age 45. The study is being done in San Francisco under a contract with Kaiser Foundation Research Institute.

Vaccination, which began in January, 1974, was completed at the end of December with over 13,000 volunteers receiving either a dodecavalent pneumococcal vaccine or a saline placebo. The vaccines have been well tolerated by the recipients and good serum antibody response to the antigens has been seen. The vaccinees will be followed for two years for development of pneumococcal disease. The Kaiser study is being performed under an independent contract; however. Dr. Austrian of the University of Pennsylvania, continues to act as consultant to this as well as other pneumococcal vaccine studies and the computer data base for the Kaiser study is maintained by him in Philadelphia.

An octavalent pneumococcal polysaccharide vaccine has been evaluated for antigenicity and toxicity in infants and children under a contract with the University of California at San Francisco which will terminate this summer. The studies have shown that the vaccines are non-reactogenic in children and give rise to good antibody response in children over two years of age. Infants a year old or younger respond less well to many of the pneumococcal antigens than do the older children.

This study has given indication of the effectiveness of this vaccine in preventing pneumococcal disease in splenectomized children and children with sickle cell disease. These individuals are particularly disposed to pneumococcal infections.

The pneumococcal vaccines used for all these studies were prepared under an expired contract with Eli Lilly.

Acute otitis media is a very important childhood disease and affects populations of all racial and social classes. Almost every child has otitis media at least once and 20% to 30% have six or more such occurrences. It is well established that pneumococci are responsible for 40% to 50% of the episodes of acute otitis media.

Acting on the recommendation of the Infectious Disease Committee, the IDB staff is preparing to initiate (before the end of FY 75) contracts to perform three studies related to otitis media in infants: (1) a trial to determine the value of the octavalent pneumococcal vaccine in preventing pneumococcal otitis media in infants; (2) a study on the safety and antibody response of infants to this vaccine with emphasis on the possible development of immune tolerance and on the determination of the proper dose and booster regimen for optimum antibody response; and (3) the development of an animal model for the study of otitis media of pneumococcal origin.

A contract was established in FY 74 with State University of New York - Downstate Medical Center, to serve as a serologic reference laboratory in support of the total IDB pneumococcal vaccine program. The laboratory, with Dr. Schiffman as Principal Investigator, has developed a new procedure for subtyping of pneumococci by radioimmunoassay (RIA) and will perform all the antibody measurements, via the RIA for the otitis media vaccine studies.

3. MYCOPLASMA CONTRACTS

A formalin-inactivated Mycoplasma pneumoniae vaccine (designated OSU-1A) has been produced under an expired contract with Ohio State University. The vaccine was tested for safety and potency by Ohio State and Dr. Senterfit at Cornell University.

A contract was initiated in FY 74 with the University of Virginia to study the clinical acceptability, antibody response, and efficacy of the OSU-1A vaccine. Following small scale toxicity and antigenicity studies, a field trial was begun in July, 1974, at the Parris Island Marine Corps Recruit Camp in North Carolina. During vaccination which ended in September, 1974, 7,861 Marine Volunteers received either the Mycoplasma vaccine or a saline placebo. The volunteers were followed for disease incidence and antibody response through their eight weeks training period at Parris Island. Approximately 10% to 20% (those assigned to Camp Lejeune after basic training) will be followed for an additional year.

Only preliminary results on the incidence of ARD and pneumonia are available at the present time. Although a significant difference in diseases between the vaccinees and controls has already been seen, the final estimate of efficacy will have to await the results of serologic assays.

The contract with Dr. Senterfit at Cornell has been maintained and he is performing a series of different serologic assays on the sera from Parris Island and Camp Lejeune. The results of the vaccine trial and the plans for the coming follow-up year were reviewed by the Infectious Disease Committee at its June, 1975, meeting.

Under a contract with Baylor College of Medicine, studies are being conducted to develop an experimental challenge system for M. pneumoniae infections. Administration of tissue-culture grown M. pneumoniae by small particle aerosol inoculation appears to produce lower respiratory disease comparable to that seen naturally. Preliminary data using this system indicate that the OSU-1A vaccine offers some protection to the M. pneumoniae challenge.

B. Methods for Other Diseases

1. STREPTOCOCCAL CONTRACTS

Group A streptococcal infections play a major part in septic pharyngitis and skin infections as well as an antecedent role in rheumatic fever and acute glomerulonephritis. An effective multivalent vaccine containing at least 6 of the predominant serotypes of M-protein would make a major impact on reducing the prevalence of these serious illnesses.

The contract with the University of Chicago has led to the development of several candidate M-protein antigens which currently are being evaluated in adult volunteers for safety and immunogenicity. The objective of this study is to demonstrate whether a vaccine composed of M-protein is efficacious against disease. Adult volunteers have been immunized intranasally with soluble type 1 M-protein and challenged with live streptococci. In contrast to previous results with parenteral immunization (in which most of the subjects exhibited significant antibody responses) intranasal immunization with M-protein evoked very poor antibody responses in both the sera and nasal washings. However, despite the lack of detectable antibody, the intranasal route of immunization prevented the subsequent colonization of the pharynx with the challenge organism. Significantly less illness and positive throat cultures were seen in vaccinees than in controls.

The studies have been extended to the type 12 streptococcal M-protein vaccine. The experimental design is similar to that used for the type 1 studies. Preliminary results indicate that type 12 M-protein is protective against challenge; however, the number of subjects involved in the experiment thus far is not adequate to draw conclusions on vaccine efficacy.

Partial data have also been obtained from a study with type 3 vaccine but here, too, the numbers are not yet adequate to determine efficacy of the vaccine.

In order to compare various methodologies for preparing pure streptococcal group A M-protein antigens, Abbott Laboratories was awarded a contract, now expired, to produce 2 grams of crude M-protein each of serotypes 1, 6, and 12. Four laboratories actively engaged in streptococcus research agreed to purify aliquots of the crude M-protein by their own methods and perform comparative tests to determine the best method for obtaining the most pure and immunogenic antigens.

Each laboratory has completed purification and aliquots of the fractions have been redistributed for chemical analysis. Arrangements are being made by IDB for production of rabbit antisera to each fraction. The sera will then be distributed to the collaborating investigators for comparative study of the immunogenic potency of the various fractions.

The current status of the University of Chicago studies and the collaborative purification program was presented before the June 13 meeting of the Infectious Disease Committee. They were asked to evaluate the results of the work, the potential for a group A vaccine, and made recommendations on the future of the group A streptococcus program.

2. SMALLPOX CONTRACTS

During a previous study to determine the best smallpox immunization procedure, using a variety of smallpox vaccines, it was found that children who were initially immunized subcutaneously and then challenged with standard calf lymph percutaneous vaccine had only a 65% incidence of neutralizing antibody on follow-up as compared to 88% of those who were initially immunized percutaneously. Cutaneous clinical response also differed depending on whether or not the vaccinee was immunized initially percutaneously or subcutaneously; those in the percutaneous group appeared to have better protection. Because of this unexpected finding, a contract has been awarded for the purpose of locating as many of the children as possible in St. Louis who received the subcutaneous smallpox immunizations. Follow-up studies will include comparisons with children who were immunized either subcutaneously or percutaneously and developed neutralizing antibody.

INDIVIDUAL CONTRACTS AND AGREEMENTS TABULATION

Program: Venereal Diseases - Venereal Disease Contracts Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-42538	University of California at Los Angeles Miller, James N.	To develop methods for the *in vitro* cultivation of *Treponema pallidum*, the causal organism of syphilis. Previous studies on the attachment and penetration of *T. pallidum* in rabbit testes tissue culture cells have been extended to an established, well-characterized human cell line. Preliminary studies on purification of the organism have suggested that the treponeme can be separated from host tissue on a ficoll density gradient.	$66,000 Renew
AI-42537	Hormel Institute - University of Minnesota Jenkin, Howard M.	To develop methods for the *in vitro* cultivation of *Treponema pallidum*, the causal organism of syphilis. A transport system has been developed to ship *T. pallidum* obtained from syphilitic lesions. Maintenance of motility of the organism has been increased by the use of pre-reduced Eagle's minimal essential medium and the *T. pallidum* has been found to attach to rat glial cells with a subsequent increase in the length of time it remains motile.	$72,000 Renew

Program: Venereal Diseases - Venereal Disease Contracts Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-42536	Stanford Research Institute Fieldsteel, A. Howard	To develop methods for the *in vitro* cultivation of *Treponema pallidum*, the causal organism of syphilis. Various types of media have been studied to determine the best for maintenance of *T. pallidum*. The contractor has developed the facility of working with the organism and growing it in rabbit testes.	Prior year funding Renew

Program: Hepatitis Program - Hepatitis Contracts Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
IA-30002	Albany Medical College Coulston, F.	To establish and maintain a breeding colony of chimpanzees. Collaborative effort of NIAID, FDA, and CDC for ensuring chimpanzees for experiments with viruses of hepatitis. Program is effective and approximately 20 newborns were produced this contract year.	$85,000 Renew
AI-22522	American Red Cross Nath, N.	To provide an Hepatitis Testing Laboratory to perform assays for hepatitis B antigens and antibodies. In addition, the testing laboratory is involved in developing better methods to test for HBV antigens and antibodies. The laboratory provides testing for other ongoing NIAID hepatitis projects, as well as services to other NIH hepatitis research projects.	$358,000 Renew
AI-32504	Meloy Laboratories Pasquale, R.	Maintain animal holding facility for hepatitis infected rhesus monkeys and chimpanzees. Essentially a service contract.	$212,000 Renew

Program: Hepatitis Program - Hepatitis Contracts Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-32509	Scripps Clinic and Research Foundation	Immunohistochemical assays of hepatitis virus-associated antigens. To develop methods for immunohistochemical assays of intrahepatic HBV associated antigens and perform pathological studies on primate liver biopsies. Methods have been developed, reagents produced, and application is being made in experimental HBV studies in primates.	$70,000 Terminate
AI-32522	State University of New York (Buffalo) Ogra, P.	To perform an epidemiologic survey of hepatitis B in a population in which viral hepatitis is highly endemic. The study encompasses the descriptive epidemiology of hepatitis B, in both patients and staff, carefully designed to document the incidence and prevalence of HB_sAg infection and illness, and the development of anti-HB_s.	$112,000 Renew
AI-12197	Tulane University Gerone, P.	To provide a facility for studying hepatitis B infections in chimpanzees. Titrations of infectious HBV pools have been completed. Future experiments will be performed with antiviral agents in the termination of the HBV carrier state and evaluation of experimental HBV vaccine.	$29,000 Renew

Program: Hepatitis Program - Hepatitis Contracts Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
IA-40005	Tulane University Gerone, P.	To establish and maintain a breeding colony of Saguinus mystax marmosets. Collaborative effort of NIAID, FDA, and CDC for ensuring future availability of small animal model for hepatitis A experiments. Field team currently in Brazil capturing and collecting breeding stock.	$53,000 Renew
AI-20002	Veterans Administration Seeff, L.	To conduct two double-blind studies administering (I.M.) either conventional low titer or high titer anti-HB_s gamma globulin to determine if these preparations lower the incidence of hepatitis B infections in certain "high risk" populations by preventing or modifying the disease. Results are unknown since studies are double-blind.	$78,000 Renew

Program: Influenza and Other Viral Respiratory Infections Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-32506	Baylor College of Medicine Couch, Robert B.	To evaluate viral vaccines for safety and efficacy in a closed population of adult volunteers. Additional studies are needed with adenovirus type 5 subunit vaccines to determine the antigenic dose of single and combined subunit vaccines necessary for obtaining the optimal serologic response. A parainfluenza virus type 3 challenge system has been developed. Reproducible ranges of B and T lymphocytes were studied in 160 normal non-ill individuals. These will be used for comparison with persons undergoing active infections. See also Disease Control Measures - Research and Development (15%), and Antiviral Substances - Antiviral Contracts (15%)	$178,000 (70%) Renew

Program: Influenza and Other Viral Respiratory Infections Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-42528	Baylor College of Medicine Couch, Robert B.	To establish and maintain an Influenza Research Center. The Center will conduct comprehensive studies of pathogenic, epidemiologic, and immunologic aspects of influenza, as well as evaluate licensed and experimental vaccines, and antiviral substances. A variety of surveillance studies designed to detect the earliest signal of influenza activity in a community, methods for detection of epidemics, and longitudinal family surveys are underway. The Houston area experienced a moderate outbreak of influenza this past winter, and the data accumulated are being compiled and analyzed. This facility was established last fiscal year and programs are being initiated and evaluated with the expectation that significant results should be available in future years.	$620,000 Renew

Program: Influenza and Other Viral Respiratory Infections Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-52517	University of California Porter, David D.	The infant ferret appears to be an appropriate animal model which develops disease similar to that seen in young infants hospitalized with RSV bronchiolitis or pneumonia. Factors which are important in the lower respiratory tract disease produced by this virus will be studied. The role of immunologic factors in resistance to RSV infection and disease as well as their possible participation in immunopathologic potentiation of disease will be defined.	$54,000 Renew
AI-42542	Case Western Reserve Univ. School of Medicine Mostow, S.	Development of an in vitro assay for attenuation of live influenza viruses. Evidence accumulated thus far suggests that influenza ts viruses, except HK/68-ts-1(A), do not inhibit ciliary activity of tracheal organ cultures at non-permissive temperatures, but completely destroy activity at permissive temperatures. Further work is needed to determine the adequacy of the tracheal organ culture for an in vitro assay of influenza virus attenuation.	$23,000 Undetermined

Program: Influenza and Other Viral Respiratory Infections Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-12091	Children's Hospital of D.C. Parrott, Robert H.	To evaluate candidate live virus vaccines for immunogenicity and safety in a closed population of infants and children. Factors which are important in the pathogenesis of viral diseases or which affect the host's resistance to infection are also studied. Long-term surveillance of vaccinees is continuing. During the past year a clinical trial of the Hong Kong strain of influenza ts-1(E) virus vaccine was evaluated. The vaccine infected 18 of 21 children with no observable illness, even in the youngest, totally seronegative children. Trials were initiated with the Udorn strain of influenza A ts-1(E) virus vaccine in children aged two to five years.	$135,000 Renew

Program: Influenza and Other Viral Respiratory Infections Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-22501	University of Colorado McIntosh, Kenneth	To conduct laboratory and clinical studies to evaluate efficacy of viral and bacterial vaccines. Comparison of administration regimens by the Red Book and by the manufacturer (Parke-Davis) of "Fluogen" (A/England/42/72 and B/Massachusetts/1/72) and "Fluogen B" (B/Hong Kong/72) were conducted. Results indicate that large doses of the influenza vaccines are well tolerated by small children. At least two doses are needed for antibody response, but the interval between doses does not appear to make a great difference. A possible association of apnea, the cessation of breathing for 20 seconds or more, with RSV infections has been observed and will be studied further. See also Disease Control Measures - Research and Development (40%)	$57,000 (60%) Renew

Program: Influenza and Other Viral Respiratory Infections Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-32521	Flow Laboratories Inc. Gilden, Raymond V.	To prepare and safety-test potential vaccine pools for the LID, NIAID, and contractors of the IDB. To provide unique tissue cultures for the LID.	$226,000 6 months Readvertise
AI-22505	University of North Carolina Denny, Floyd W.	To conduct studies to test the safety, antigenicity, immunogenicity, and person-to-person spread of attenuated respiratory vaccines in children. These studies include respiratory syncytial virus (RSV) ts-1 mutant, and the Hong Kong strain of influenza ts-1(E) vaccine. Studies of the behavior of natural acute respiratory diseases in defined populations have continued. Recent studies have defined patterns of acquisition and carriage of specific pneumococcal serotypes and new insight on the pathogenesis of Mycoplasma pneumoniae disease has been developed. During this final year the extensive study of RSV infection and disease will be completed.	$58,000 Terminate
AI-42543	Public Health Research Institute of the City of New York, Inc. Pons, Marcel W.	To search for evidence that all or a portion of the genome of influenza virus, RSV, or parainfluenza type 3 virus persists in the cells of the respiratory tract of man either in the form of viral RNA or as complementary DNA. Contractor has developed methodologies for the preparation of highly specific and sensitive nucleic acid probes for influenza genetic material. Techniques for developing the RSV probes are being pursued.	$50,000 Renew

Program: Influenza and Other Viral Respiratory Infections Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-22303	University of Rochester Douglas, R. Gordon	To clinically evaluate viral vaccines as they become available (live and inactivated influenza, live RSV,live and inactivated parainfluenza viruses). A live influenza recombinant, A/Udorn/307/72 ts-1(E),was tested in forty volunteers. Various titers of virus inoculum were employed. A wild type rechallenge was performed in 11 vaccinees and 3 placebo subjects. Febrile illness occurred in 1 placebo subject, but in none of the vaccinees. The vaccine was shown to be safe, infective, antigenic, and efficacious, even at the highest dose of virus used. An inactivated commercial bivalent vaccine was administered to 29 patients who had undergone renal transplantation. Ten of 13 patients with normal renal function developed 4-fold or greater antibody rises to either influenza A or B, whereas 6 of 17 with compromised renal function did not. In addition, 29 patients on chronic maintenance hemodialysis were studied. Only 11 responded to influenza A antigen, and 6 to B. The magnitude of response was clearly lower than that observed among normals. Also see Disease Control Measures - Research and Development (25%)	$120,000 (75%) Renew

Program: Influenza and Other Viral Respiratory Infections Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-22504	Rutgers University Simpson, Robert	To develop attenuated mutants of RSV and parainfluenza viruses as potential vaccines. During the past year it was demonstrated that RSV can establish chronic persistence in HEp-2 and BEK cells. Additional evidence suggests that this RNA virus can enter into association with host cells in the form of a proviral DNA intermediate.	$107,000 Renew
AI-42509	Rutgers University Simpson, Robert	To evaluate influenza temperature sensitive mutants for phenotype, genetic defects, and infectious potential. Plaquing characteristics and complementation analyses of mutants being conducted.	-0- (prior yr) Undetermined
AI-42510	St. Jude Children's Hospital Webster, Robert	To isolate and purify various polypeptide antigens of influenza virus for evaluation as subunit vaccine. Results of a subunit vaccine preparation inoculated into volunteers indicated that combined subunits (HA, NA, and RNP) were as antigenic as whole inactivated vaccine preparation. Further purification of the subunits is in progress.	$6,000(prior yr) Undetermined

Program: Influenza and Other Viral Respiratory Infections Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
IA-50003	St. Louis University Green, Maurice	To analyze human cancers for copies of gene sequences specific for human adenovirus types 2, 7, and 12. With the use of highly specific and sensitive nucleic acid probes adenovirus DNA has been detected in normal and diseased tissues.	$10,000 Renew
AI-42511	State University of New York (Buffalo) Ogra, Pearay	To conduct a clinical field trial with X41 (H Port Chalmers/N Port Chalmers), X42 (H equi 1/N Port Chalmers) inactivated influenza virus vaccines and placebo. The vaccines have been administered to 1,000 school children (ages 9-14): 400 X41, 400 X42, and 200 placebo. The vaccines were shown to be non-reactogenic and antigenic. Buffalo experienced outbreak of Port Chalmers influenza A this past winter, so efficacy data should be obtained in the future.	-0- (prior year) -0- (prior year)

Program: Influenza and Other Viral Respiratory Infections Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-22507	Vanderbilt University Karzon, David T.	To conduct clinical evaluations of respiratory virus vaccines in infants and children. RSV ts-1 mutant vaccine was evaluated in 25 children in sequential trials. No clinical illness could be associated with virus shedding. Natural RSV reinfection occurred in 6 of the 8 vaccinees who had been infected with RSV ts-1. The rate of reinfection and the types of illness seen with reinfection appeared similar between controls and vaccinees. During the past year influenza A ts-1(E), Hong Kong strain, was evaluated in doubly seronegative children. Eighteen vaccinees and 6 controls were studied in five trials. All of the children who received $10^{3.5}$ $TCID_{50}$ or greater of influenza ts-1(E) were infected. Revertant virus was isolated from 4 of 14 children shedding virus. The revertant virus showed a tendency to become the dominant population. In spite of reversion and high titers of virus shed, no transmission of influenza ts-1(E) to the controls occurred, nor was there any conclusive evidence that it caused illness. A moderate HAI titer was achieved in the 16 of 18 vaccinees with an HAI response. Response of anti-neuraminidase antibody was varied.	$234,000 Renew

Program: Influenza and Other Viral Respiratory Infections Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-22508	University of Virginia Gwaltney, Jack M.	To clinically evaluate live attenuated influenza virus vaccines administered by the nasal route in appropriate adult populations to show infectivity, degree of virulence, and efficacy. A live influenza recombinant, A/Udorn/307/72 ts-1(E) was administered to twenty college student volunteers. The vaccine was shown to be safe, infective, and antigenic.	$58,000 Terminate

Program: Antiviral Substances - Antiviral Contracts Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-22532	University of Alabama Ch'ien, Lawrence T.	To clinically evaluate 9-β-D-arabinofuranosyl adenine against herpes encephalitis, disseminated herpes zoster and serious herpes hominis infections particularly in immunologically compromised patients. This is an extensive collaborative program involving approximately 20 clinics. The double-blind study is progressing well.	$283,000 Renew
AI-32506	Baylor College of Medicine Couch, Robert B.	To evaluate chemoprophylactic and chemotherapeutic agents against human respiratory virus infections. Interferon response in seronegative and seropositive volunteers is being determined following intranasal administration of live rubella virus vaccine. See also Influenza and Other Viral Respiratory Infections (70%) and Disease Control Measures - Research and Development (15%).	$38,000 (15%) Renew

Program: Antiviral Substances - Antiviral Contracts Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-42530	Baylor College of Medicine Greenberg, Steven B.	To continue studies to determine why large amounts of interferon when administered intranasally are needed for effective inhibition of rhinovirus whereas doses four orders of magnitude smaller are totally inhibitory in human tissue culture, and to design ways to decrease by several orders of magnitude the dosage required for effective viral inhibitions.	Prior year funding Renew
AI-32520	Lee-Mac McCoy, Thomas A.	To apply a unique cytogenerator to the production of human interferon. The contractor has successfully applied the cytogenerator to the purposes indicated.	Terminate
AI-52521	Lee-Mac McCoy, Thomas A.	Using optimal induction procedures, this contractor will design and test hardware for large-scale production of human interferon. These techniques will then become available to producers of clinical material.	$70,000 Renew
AI-02169	New York University Medical Center Vilcek, Jan T.	To develop a more economically feasible method for the production of human interferon which would be acceptable for use in humans. This contract also produced human interferon for purification studies.	$84,000 Renew

Program: Antiviral Substances - Antiviral Contracts Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-02126	Medical College of Pennsylvania Paucker, Kurt	To develop potent and specific antisera to murine and human interferon and to produce crude and partially purified mouse interferon for use by other contractors. Reagent quantities of mouse and human interferon antisera are now available as is the mouse interferon. The goals of this project have been satisfactorily met and the contract has been terminated.	Terminate
AI-52520	Medical College of Pennsylvania Paucker, Kurt	To develop monospecific antibodies against human leucocyte and fibroblast interferons. This will be accomplished by induction of cells to produce antisera to interferon, mutagenizing cells and selecting those which are producing only one antibody, that directed against interferon. This material will be useful in interferon purification and mechanism studies.	$58,000 Renew

Program: Antiviral Substances - Antiviral Contracts Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-32529	University of Pittsburgh Youngner, Julius S.	To develop a biological interferon inducer which is effective and nontoxic with clinical potential. Established biological inducers will be compared and most effective ones chemically characterized. The three phases include: accumulation and purification, comparison and evaluation and identification of the active center. This center will work on Salmonella, Brucella and Xanthomonas organisms.	Prior year funding Renewal dependent on current results
AI-42527 (32501)	Stanford University Merigan, Thomas C.	To clinically evaluate the role of exogenous interferon against serious varicella viral infection in immunologically compromised patients. The initial studies to evaluate the possible toxicity of interferon are completed and the double-blind study is making excellent progress.	$92,000 Renew

Program: Antiviral Substances - Antiviral Contracts Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-32530	Medical College of Wisconsin Grossberg, Sidney E.	To develop a biological interferon inducer which is effective and non-toxic with clinical potential. Established biological inducers will be compared and most effective ones chemically characterized. The three phases include: accumulation and purification, comparison and evaluation and identification of the active center. This contractor will work on E. coli proteins as well as other bacterial and viral products.	Prior year funding Renewal dependent on current results
AI-42514	Medical College of Wisconsin Grossberg, Sidney E.	To lyophilize rabbit interferon and prepare in ampule form as a research reference reagent; this includes stability studies and periodic verifications of titer.	$48,000 Terminate
AI-42520	Medical College of Wisconsin Grossberg, Sidney F.	To study partially purified human interferon in an attempt to stabilize it. Such studies are of importance since interferon loses its titer as it is purified. If this material is to be used clinically, methodologies for its stabilization should be found.	$64,000 Renew

Program: Antiviral Substances - Antiviral Contracts Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-02130	Washington State University Duenwald, Jeff C.	This contract was terminated by mutual agreement. The investigator could not devote the time that was required for satisfactory accomplishment of the workscope.	Terminated
AI-42524	University of Utah Glasgow, Lowell A.	To provide a viral disease animal model which closely mimics a disease state in man so as to allow evaluation of promising antivirals as soon as they are identified. This contract is a component of the Cooperative Antiviral Testing Group which tested several antivirals and continues baseline studies with interferon and its inducers. This particular contract includes herpesvirus, cytomegalovirus and EMC virus in the mouse.	$99,000 Renew
AI-81266	New York Medical College Galin, Miles	Component of the Cooperative Antiviral Testing Group. This contractor is studying viral infections (herpes) of the rabbit eye.	$38,000 Terminate
AI-02131	New York University Medical Center Friedman-Kien, Alvin E.	Component of the Cooperative Antiviral Testing Group. This particular contract includes vaccinia, fibroma and herpesviruses as dermatological infections in the mouse and rabbit.	$85,000 Renew

Program: Antiviral Substances - Antiviral Contracts Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-42523	University of Utah Janis, Burton	Component of the Cooperative Antiviral Testing Group. This particular contract includes studying the rabies virus model in the mouse.	$69,000 Terminate
AI-02132	University of Wisconsin Easterday, Bernard C.	Component of the Cooperative Antiviral Testing Group. This particular contract includes swine influenza, transmissible gastroenteritis and pseudorabies viruses in the piglet.	$59,000 Renew

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-32506	Baylor College of Medicine Couch, Robert B.	To evaluate an inactivated *Mycoplasma pneumoniae* vaccine (OSU-1A) by developing and testing experimental infectious challenge systems for *M. pneumoniae* following immunization. Results have shown that the OSU-1A vaccine was relatively non-reactogenic, was antigenic and that serum antibody titers were not enhanced following booster dose. The vaccine was found to be stable during storage at 4 C. See also Influenza and Other Viral Respiratory Infections (70%) and Antiviral Substances - Antiviral Contracts (15%)	$38,000 (15%) Renew
AI-52502	Central Public Health Laboratory Mäkelä, P. Helena	To conduct an efficacy trial of the meningococcal group A polysaccharide vaccine in children 3 months to 6 years of age using the *Hemophilus influenzae* polysaccharide vaccine as a control. Approximately 50,000 children have received each of the vaccines in three counties in Finland. The vaccines have been well tolerated. The vaccinees and a control unvaccinated population are being followed for incidence of meningococcal and *H. influenzae* disease. Conclusive efficacy data is not yet available.	$134,000 Renew

Disease Control Measures - Research and Development   Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-12196	Children's Hospital of Boston Anderson, Porter	To conduct clinical evaluation of *Hemophilus influenzae* b polyribose phosphate (PRP) vaccine in infants, to serve as a serologic reference laboratory, and to develop alternate candidate vaccines for *H. influenzae*. Results have shown that antibody response to PRP in infants is poor. A new method of purification of the vaccine has yielded a new form of PRP (designated PRP-X) which has been shown to be immunogenic in weanling mice and may be a promising new vaccine preparation for immunization of infants.	$127,00 Renew
AI-02065	Cornell University Senterfit, Laurence B.	To serve as a serologic testing laboratory for the study of the antibody responses of volunteers in the University of Virginia/Parris Island *Mycoplasma pneumoniae* vaccine trial. Sera from the Parris Island trial has been sent to Cornell and several different assays for *Mycoplasma* antibody are being performed. Assays will be performed on follow-up sera from Camp Lejeune.	$26,000 Terminate

Disease Control Measures - Research and Development   Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-22500	University of California Ammann, Arthur	To determine optimum dosage of octavalent pneumococcal polysaccharide vaccine for maximum antibody response in infants and children. Results suggest that immunization of infants greater than 1 year of age can be accomplished with safety and will result in measurable antibody response. Children under 1 year of age respond poorly to most of the individual polysaccharides in the octavalent vaccine. Variations in dosage and use of boosters does not improve the response in infants.	0 Terminate
AI-52523	University of California at Los Angeles School of Medicine Cherry, James D.	To study various parameters of immune response in children who received smallpox immunizations subcutaneously which were followed by percutanous smallpox immunizations which did not result in a "take" in the vaccine recipient.	$125,000 (15 months) Terminate

Disease Control Measures - Research and Development   Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-32519	University of Chicago Fox, Eugene N.	To determine the effectiveness of streptococcal M-protein vaccines in prevention of infection in human challenge experiment. Results indicate that parenteral and intranasal immunization with type 1 M-protein protects against type 1 challenge. Preliminary experiments suggest a similar protective effect of the type 12 and type 3 vaccines.	$94,000 Renew
AI-22501	University of Colorado McIntosh, Kenneth	To conduct clinical evaluation studies of *Hemophilus influenzae* b polyribose phosphate (PRP) and meningococcal groups A and C polysaccharide vaccines in children. Results indicate that children under 2 years of age respond less well than older children to the *H. influenzae* vaccine. Likewise, the response of younger children to the meningococcal vaccines appeared to be less than the response of older children. Studies on antibody response to *H. influenzae* disease and carriage have indicated that antibody response to disease is variable and that carriage in the nasopharynx following disease is immunogenic. See also Influenza and Other Viral Respiratory Infections (60%)	$38,000 (40%) Renew

Disease Control Measures - Research and Development Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Development	FY75 Obligations FY76 Plans
AI-22502	University of Connecticut Gold, Ronald	To conduct clinical evaluation studies of meningococcal group A and C polysaccharide vaccines in infants. No significant local or systemic reactions have been observed with the vaccines. Optimum dose level of group C vaccine for primary immunization was shown to be 100 ug. Booster inoculations with C vaccine were not effective when given at 7 and 12 months following initial dose at 3 months. Booster inoculations are effective with the group A vaccine and result in significant antibody responses in infants.	$71,000 Renew
AI-32517	University of Pennsylvania Austrian, Robert	To conduct studies on the epidemiology and immunology of pneumococcal infection and to conduct a field trial to demonstrate efficacy of candidate multivalent pneumococcal polysaccharide vaccines. To perform serologic assays on sera from the trial conducted under subcontract and on sera from the Kaiser Permanente efficacy trial (NIAID Contract). Vaccination of 1300 volunteers at Dorothea Dix Hospital (subcontract) has been completed and disease follow-up is in progress. Preliminary data indicate the vaccine will be effective in preventing type specific pneumonia. Technical assistance is provided by this contractor to the Kaiser field trial (supported by NIAID) and to field trials in South Africa (not supported by NIAID).	$297,000 Renew

Disease Control Measures - Research and Development   Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-22513	Kaiser Permanente Fried, Marvin A.	To conduct a field trial to determine the efficacy of a dodecavalent pneumococcal polysaccharide vaccine in preventing type-specific pneumococcal pneumonia in adults age 45 and older. More than 13,000 volunteers have received either the vaccine or a saline placebo. No serious reactions have been observed and antibody response to all the monovalent components has been good. Follow-up for incidence of pneumonia is in progress; no conclusive data on efficacy is yet available.	$241,000 Renew
AI-12502	Rockefeller University Gotschlich, Emil C.	To serve as a reference testing laboratory to measure (using the radioimmunoassay procedure) the serum antibody response of children and infants to immunization with groups A and C meningococcal polysaccharide vaccines. Assay of serum specimens from IDB meningitis vaccine contractors is ongoing.	$30,000 Renew

Disease Control Measures - Research and Development   Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-22303	University of Rochester Douglas, R. Gordon	To conduct clinical studies to evaluate reactivity and antigenicity of candidate bacterial vaccines. Hemophilus influenzae type b polyribose phosphate (PRP) is being evaluated in infants (younger than 6 months) and polyvalent pneumococcal vaccines in adults over the age of 50. Results indicate that the serum antibody response in children in the 14-18 months age group is less than that in the 2-4 year age group. In general, pneumococcal polysaccharides have been demonstrated to be nonreactogenic in elderly patients and the frequency of antibody response in all cases is high. See also Influenza and Other Viral Respiratory Infections (75%).	$42,000 (25%) Renew
AI-22506	University of Southern California Wehrle, Paul F.	To conduct clinical evaluation studies of the Hemophilus influenzae type b polysaccharide and meningococcal A and C polysaccharide vaccines in infants. To set up a disease surveillance system and study the incidence of bacterial meningitis in Los Angeles County. Results indicate a poor response by infants to the H. influenzae vaccine and low but measurable antibody responses to meningococcal groups A and C vaccines. The disease surveillance system is just being organized. No data are available.	$104,000 Renew

Disease Control Measures - Research and Development   Branch: IDB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-42541	State University of New York (Downstate Medical Center) Schiffman, Gerald	To serve as a serologic testing laboratory in support of the total pneumococcal vaccine program. In particular, to support studies on safety and antigenicity of pneumococcal polysaccharides in infants and efficacy of an octavalent pneumococcal vaccine for prevention of otitis media in infants. The contractor measures antibodies to pneumococcal polysaccharides using the radioimmunoassay procedure. A method for subtyping pneumococci by the radioimmunoassay has been developed.	$83,000 Renew

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Obligations FY76 Plans
AI-42512	University of Virginia Wenzel, Richard P.	To determine the efficacy of a formalin-inactivated *Mycoplasma pneumoniae* vaccine (OSU-1A) in preventing *Mycoplasma* pneumonia. Approximately 8,000 volunteers at the Parris Island Marine Corps Recruit Camp received either the OSU-1A vaccine or a saline placebo in the summer and fall of 1974. The vaccine was well tolerated by the volunteers and disease incidence and serologic data are currently being analyzed. Indications are that the vaccine has been of value in reducing the disease but conclusive efficacy data are not yet available. Follow-up is continuing at Camp Lejeune, North Carolina of those Marines transferred there for duty.	$44,013 Terminate

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INFECTIOUS DISEASE BRANCH WORKSHOPS FOR PROGRAM REVIEW, DEVELOPMENT OR EVALUATION

Date	Meeting	Purpose
June 10, 1974	A workshop was held on "Disease Accentuation after Immunization with Inactivated Microbial Vaccines". Included in the discussions were the results of the inactivated RSV and *Mycoplasma pneumoniae* vaccine trials which were carried out under NIAID sponsorship.	Program Review and Development
July 15, 1974	A workshop was held to review the problem of nosocomial infections and to discern if anything could be done to help solve the problem through the contract research mechanism. Particular emphasis was placed on current studies on gram-negative bacterial antigens and their potential as vaccines.	Program Development
September 13, 1974	A workshop was held entitled "Infections with Cytomegalovirus in Bone-marrow Transplantation".	Program Development
October 10, 1974	The Infectious Disease Committee reviewed the Smallpox Vaccine Program.	Program Review, Development and Evaluation
October 11, 1974	The Infectious Disease Committee reviewed the problem of hospital associated infections and made recommendations as to possible future program activities.	Program Development
December 6, 1974	A meeting was held to discuss the possibility of clinical trials of ara-A and disodium phosphonoacetate in cutaneous herpes simplex virus infections.	Program Development
December 12, 13, 1974	Influenza workshop on Antivirals for Influenza	Program Development

IDB Workshops (cont.)

Date	Meeting	Purpose
December 16, 17, 1974	A workshop was held on the use of pneumococcal polysaccharide vaccines in infants.	Program Review and Development
January 16, 1975	The Infectious Disease Committee reviewed the hepatitis program.	Program Review, Development and Evaluation.
January 17, 1975	The Infectious Disease Committee reviewed the clinical studies on adenine arabinoside against herpes infections.	Program Review, Development and Evaluation
June 12, 1975	The Infectious Disease Committee reviewed the *Mycoplasma* Vaccine Program.	Program Review, Development and Evaluation
June 13, 1975	The Infectious Disease Committee reviewed the Streptococcal M-Protein Vaccine Program and discussed other streptococcal disease areas.	Program Review, Development and Evaluation

GMB

LSD

L&CR

LCI

LID

GEOGRAPHIC MEDICINE BRANCH

The Geographic Medicine Branch was created on 1 July 1968 to manage several biomedical programs transferred to the National Institute of Allergy and Infectious Diseases (NIAID) from the Office of International Research. The Branch is currently part of Collaborative Research Programs, NIAID, and consists of two principal organization elements: The U.S.-Japan Cooperative Medical Science Program Section and the International Centers for Medical Research Section (ICMR).

Grants and contracts are used in support of U.S.-Japan program, but grants are the only support instrument for the International Centers. For a detailed history of these programs and their operating methods, the reader is referred to the NIAID Annual Report for FY73 page 1 through 14.

The Joint Committee and the seven Program Panels meet annually, alternating between the United States and Japan. This year all of the meetings will be held in the United States. At the Joint Committee meeting, October 30 and 31, 1975, Program reviews of the Leprosy and Parasitic Diseases Panels will be initiated, and the final draft of the Second Five Year Report will be considered.

I. Disease Control Measures - Research and Development
U.S.-Japan Cooperative Medical Science Program

1. CHOLERA

A field trial employing a highly purified cholera toxoid began in Bangladesh in July of 1974. Two injections of 100 mcg each were given at six-week intervals to approximately 46,000 individuals. Adult-type tetanus-diphtheria toxoid was administered as a control to another 46,000 in the same community.

The incidence of El Tor cholera began in July,1974,and reached a peak in October. The epidemic was unusually severe which provided an excellent opportunity to assess the protective level of the toxoid. An analysis of the data for all age groups indicated that toxoid conferred about 40 percent protection during the first nine weeks and no significant protection during the ensuing months of observation. When the protective effect was examined by age group and serotype, however, it appeared that this toxoid conferred higher protection in children under age four. For example, in the one to four age group, protection against being hospitalized with Inaba cholera was as high as 70 percent during the first two months. This observation has added significance since cholera is largely childhood disease in Bangladesh. In general, individuals who received two injections did not enjoy greater or more prolonged protection than those who received only one.

It is clear from the early results of this trial that this purified toxoid in the dose used did not afford the duration of protection which would be useful in practical cholera control; however, the trial did demonstrate that a parenterally administered toxoid can confer a limited degree of transient protection. In this sense, the trial has already provided at least a partial and affirmative answer to a question asked: Does antitoxin immunity play any role in the resistance to cholera?

Further work should attempt to determine how best to enhance the limited protection which has been seen in this first field trial. The ultimate aim is to determine which combination of somatic and toxoid antigens administered by which route will provide a more effective and long-lasting immunoprophylactic agent against cholera. Lastly, but very important, the successful completion of this trial, which involved a very large number of people, is a momentous achievement in itself. The mechanics, the presonnel, and the expertise are available for conducting future trials of a similar nature.

Studies of oral administration of toxoid to dogs indicates local antitoxic immunity offers more protection against challenge doses of V. cholerae than circulating antitoxic immunity. Extensive work in this area is being done as another avenue of producing a more effective cholera immunogen. At the same time, data coming from these studies in dogs indicates there may be a loss in protection from a toxoid prepared by sartorius filtration to remove the somatic antigen. This phenomenon is also being investigated.

Further studies with zirconyl oxychloride as a means of stopping fluid loss in cholera revealed that the beneficial effect was due to the sudden change in pH of the intestine. The search continues for chemicals which may counteract the fluid loss caused by cholera toxin.

The U.S. and Japanese Cholera Panels have initiated a cooperative venture between the two countries to standardize the characterization of noncholera vibrios. Two laboratories in each country are involved. The plan is to share type-cultures from each country's collection and compare biochemical and serological results with the idea of arriving at a mutually agreeable classification system. The United States has sent 86 type-cultures to Japan, and has received 38 type-cultures in return. Early results indicate that 31 of the Japanese cultures may be similar to U.S. type-cultures.

Studies on the incidence of diarrhea caused by enterotoxin producing E. coli are progressing well. The first 40 cases of diarrhea studied in a pediatric hospital in Brazil indicates as many as 50 per cent of the children had lactose-fermenting organisms that produced the cholera-like, heat-labile toxin as the only fecal pathogens. So far, very few E. coli have been found that produce only heat-stable toxin. Thus, the importance of strains that produce only heat-stable toxin in human disease remains unknown. The role of enterotoxigenic E. coli should come into better focus as more cases are added to the study. Material from these E. coli studies is being shared with Dr. Albert Kapikian at NIH for studies on the frequency of detection of the reovirus-like agent associated with some infantile diarrheas.

2. ENVIRONMENTAL MUTAGENESIS AND CARCINOGENESIS

The Environmental Panel is the newest component of the U.S.-Japan Cooperative Medical Science Program, having been added in 1972. The NIEHS supports and monitors the contracts and grants assigned to this Panel while the NIAID organizes and funds essential workshops and conferences and coordinates all administrative program activities. The National Cancer Institute also maintains an active and mutual interest in the goals and objectives of this program, and has provided partial travel support for co-sponsored workshops. The Panel's diversified membership has resulted in a liaison among the NIEHS, NCI, NIAID and other segments of the scientific community.

This year the Environmental Program initiated a substantive research effort to develop and validate selected bioassay systems. A systematic study is being made of the dominant lethal method and of the tests for the induction of chromosomal damage to determine if these methods are really diagnostic for detecting transmissible genetic damage in the form of gene mutations and viable translocations. Dominant lethal mutations consist in part of open chromosome breaks and in part of a symetrical exchange. In Drosophila, where both of these types of damage can be measured as well as heritable translocations and gene mutations, the frequencies with which different categories of genetic damage are induced in a variety of germ cell stages by representative chemical mutagens are being compared. The outcome of these investigations will be of value in determining whether the results of the dominant lethal test have more general validity.

Research is underway to develop a new mouse strain to maximize the sensitivity of a point mutation assay. The sensitivity of a point mutational assay system is directly related to the number of loci that can be tested in each individual. In the present experimental mouse strains (DBA/2J or C57BL/6J) nine loci, which control the electrophoretic mobility of various enzymes, can be sampled. In other mouse strains which have been studied, 13 additional electrophoretic variants have been identified. Initial crosses of these mutants to the C57BL/6J have been studied with the ultimate aim of developing a new strain homozygous with C57BL/6J.

Another research effort is directed toward developing a system to detect recessive lethal mutations in mice. The object is to develop strains of mice carrying inversions which can be used to assay for gene mutations in the chromosome regions which they cover.

To assist in monitoring the human population, three assay systems are being developed: (1) a simple assay on human red blood cells to detect fetal hemoglobin believed to be the result of mutation: (2) adaptation of the Tradescentia test system to chemical and environmental studies: (3) the ultization of diploid human fibroblasts in tissue culture to assay for gene mutations at the HGPRT and AGPRT loci.

The Environmental Mutagen Information Center continues to review the current literature and all published reports on tests for chemical mutagenesis and to incorporate pertinent information in its bibliographic files. Keywording with respect to agent, test organisms and test object and a summary table of the data are being included in the data bank.

In collaboration with Japanese scientists, a joint U.S.-Japan workshop on "The Mutagenicity of Chemical Carcinogens" was held to review the status of on-going programs in the United States and Japan in which large numbers of carcinogens and non-carcinogenic structural analogs are being tested for mutagenic activity. The results of testing 152 chemicals were presented. Marked differences were found with the different assay systems, both with regard to the total number of compounds successfully tested as well as the correlation between carcinogenic and mutagenic activity. It was clear that many chemical carcinogens required metabolic activation to be mutagenic and that activation was possible using *in vitro* tests with liver microsome preparations. It was also noted that the highest correlation was obtained with the microbial assay systems (mutation-induction in Salmonella, DNA repair in *E. coli* and *B. subtilis*) with *in vitro* metabolic activation. Plans were made to test all 152 compounds with these assays.

3. LEPROSY

A variety of immunologic studies have been carried out during the past year. Immunofluorescent "banding" of the dermal-epidermal junction of skin was demonstrated in three of seven patients with lepromatous leprosy by direct immunofluorescence microscopy. The "banding" was caused specifically by deposition of IgM. Within the glomeruli of one patient, dense, amorphous deposits in subendothelial and intramembranous position were also demonstrated by electron microscopy. These preliminary finding suggest that lepromatous leprosy may be associated with immunologic disturbances of both skin and glomerular basement membranes.

Qualitative and quantitative analyses of the local exudative response to skin abrasion were performed on patients with tuberculoid and lepromatous leprosy and compared with control subjects. The inflammatory response of leprosy patients was qualitatively normal as measured by serial coverslip preparations over a period of 28 hours. Migratory sequences and proportions of polymorphonuclear leukocytes (PMN), lymphocytes and macrophages did not differ from controls throughout the 28 hour period of study. Application of *M. leprae* antigens to abrasion sites at 12 hours induced a transient PMN migration into the exudates of all subjects. The response was greatest in patients with tuberculoid leprosy and minimal among patients with lepromatous disease.

Mobilization of leukocytes into cell collection chambers from skin abrasions was significantly decreased among patients with lepromatous leprosy as compared with controls. It is suggested that humoral or tissue factors may be present in patients with lepromatous leprosy that may alter the kinetics of PMN responses to inflammatory stimuli.

In studies on defective leukotoxis and inflammation in leprosy, sera from patients with leprosy were examined for the presence of leukotactic inhibitors. Assays were performed using human neutrophils, chambers with micropore filters and chemotactic factors consisting of fragments of the third and fifth components of complement as well as the bacterial chemotactic factor. Sera from 17/20 patients with lepromatous leprosy contained an inhibitor that inactivated the chemotactic factors in contrast to 10 normal sera that had no inhibitory activity. Of these 17 patients, 12 were anergic (lack of dermal reactivity to candida, mumps, trichophytin and streptokinase-streptodornase antigens). In three additional patients inhibitor was present in the face of questionable anergy, while in five other patients anergy was not associated with an inhibitor. Addition of diaminodiphenylsulfone in pharmacological levels to serum failed to result in leukotactic inhibition indicating that chemotherapy was not related to the leukotactic defect. The leukotactic inhibitor in leprosy serum has features of the chemotactic factor inactivator and may be responsible, at least, in part, for impairment of cellular inflammatory responses in leprosy.

In January 1975, the clinical trials to evaluate chemotherapeutic agents in Filipino leprosy patients were resumed when a new contract was awarded to the Leonard Wood Memorial Foundation. There are indications that the long term studies initiated prior to the break in contract continuity are intact, and it is anticipated satisfactory progress will be made during FY 76.

The fourth annual surveillance of post-treatment of leprosy patients among the Pingelapese population was completed in January-February 1975. Of the 2103 membered study population, 1597 received an annual leprosy examination. The re-examination rate was estimated to be 94.5%. Seventy-one individuals of the original examination roster have died since 1968 and none of these had developed leprosy prior to the time of death.

Presently the investigators conclude that eradication of leprosy in a population by use of DADDS for preventive treatment cannot be effective without simultaneous and continuous drug control of all bacilliferous cases in the population. Studies will continue in FY 76 to determine if mass treatment with DADDS is associated with the emergence of sulfone resistant infections.

Excellent progress has been made in preparing Transfer Factor to be used in a controlled clinical trial to evaluate the immunotherapeutic response of this agent in the treatment of lepromatous leprosy. To date, 75 doses of Transfer Factor from Dharmendra positive donors have been prepared and 100 doses of Transfer Factor from coccidioidin positive donors have been prepared. It is anticipated that the remaining doses of Transfer Factor will be prepared by July 1975, after which it will be tested for activity in normal recipients.

Studies on the _in vitro_ cultivation of _Mycobacterium lepraemurium_ were initiated during the past year. Preliminary work has demonstrated that fetal calf serum is superior to goat or bovine sera as a supplement to Nakamura's basal medium. A thorough evaluation of the efficiency of different sera is in process. Other factors such as microaerophilia and levels of aerobiasis are under investigation to determine methods and techniques to enhance _in vitro_ cultivation of _M. lepraemurium_.

New knowledge accumulates from pharmocologic studies. A number of salicylic acid derivatives were screened, in the hope that one of these might be used by the _M. leprae_ to synthesize a spurious "mycobactin"; one -- 3,5-diisopropylasalicylic acid -- previously screened and found to be minimally active, is planned.

Two drugs previously found active against M. leprae were restudied. 4-amino-4'-hydroxy DDS was found to be active by virtue of its contamination with DDS, evidently a by-product in the synthesis. A lederle-produced anti-schistosomal agent, (an imidazolylthiadiazole) was found to be highly active when administered in the mouse diet in a concentration of 0.2g%, and inactive at concentrations ranging downward from 0.1g%.

Studies were performed with tilerone which is a small molecule (M.W. - 400) that functions both as an inducer of interferon and as a non-specific stimulant of cell-mediated immunity in a variety of animal models. Pilot studies have shown that tilerone administered in the diet inhibits multiplication of M. leprae in the mouse foot pad. The results, however, did not distinguish between a direct antimicrobial effect or an enhancer of cell-mediated immunity. RMI 11,002 DA, an analog of tilerone, was less active. Further studies are planned.

Excellent progress has been made with the armadillo animal model for leprosy research. It has been found that infected armadillos will provide M. leprae in sufficient quantity to prepare an abundance of lepromin, a reagent used for the diagnosis of leprosy. Lepromin prepared from armadillo tissues has been tested on 74 patients in the Republic of Zaire and found to give the same reaction as lepromin prepared from human tissues.

In addition techniques have been developed for harvesting M. leprae from liver and spleen aseptically. The armadillo liver and spleen have been shown to be excellent sources of M. leprae, containing 10^{10} to 10^{12} bacilli per gram. Livers and spleens of infected animals weigh approximately twice as much as those of normal animals.

To provide a stimulus to research on the immunochemistry of M. leprae, Program staff plans to supply qualified investigators with working quantities of organisms harvested from infected armadillos.

4. MALNUTRITION

The Malnutrition Program crosses Institute organizational lines by involving NIAID, NIAMDD and NICHD. Since NIAMDD had the required expertise in the nutritional area, initially it was charged with the responsibility of managing the grants program while NIAID supplied the funding. In 1970, it was considered administratively desirable to transfer $1,023,000 in NIAID grant funds to the NIAMDD for continuation of this support. Since the Malnutrition Program guidelines also involve pediatric research, the NICHD provides a portion of the necessary expertise to successfully monitor these grants, and the NICHD shares the grant base allocated to the NIAMDD. Contracts are reviewed by the Malnutrition Panel and appropriate Institute and are currently funded by the NIAID. In addition, the NIAID organizes and supports essential workshops and conferences and coordinates all administrative program activities.

During the past year the Malnutrition Program has placed more emphasis on the relationship between malnutrition and immune response to infectious agents. This trend is reflected in the conference contract with the Massachusetts Institute of Technology which provided partial support for the "Workshop on Immune Status in Malnutrition" held at the KROC Foundation, California on May 19-23, 1975.

The six major subjects covered at this important workshop included:

1) biology of immunoglobulins (humoral and secretory); 2) cell mediated immune response in malnutrition; 3) vaccination response in the malnourished child; 4) the role of the complement system in malnutrition; 5) phagocytosis and macrophage activity in the malnourished individual; and, 6) the role of other non-specific factors including vitamins, minerals, and hormones.

It is anticipated the most recent knowledge and advances in these areas will be prepared into final proceedings and position papers by the Subcommittee on Nutrition and Infection of the National Academy of Sciences in collaboration with members of the Malnutrition Panel.

Investigators at the National Institute of Nutrition, Mexico City, Mexico have continued studies on the role of lactation in the nutrition of low socio-economic groups. A two year longitudinal study was made of the production and consumption of 17 mother-child units in a poor and inadequately nourished rural community. A comparable group was similarly studied with the exception that the diet of the mother was supplemented from the 45th day of gestation until weaning. The children of these mothers were also given food supplements, vitamins and minerals from the third month of age onwards. It was found that in the first six months of lactation, the consumption of milk by infants in the non-supplemented group followed a curve with a maximum peak of 650 ml per day. After the sixth month, there was a tendency towards a plateau of about 450 ml. In supplemented mothers, the curve for the first semester was smoother and the volume secreted was 15 percent higher. There was also a decrease in production after the sixth month. It was also found that the milk of the supplemented mothers was more dilute, but it did not differ significantly in its total solid content from the milk produced by the non-supplemented mothers.

During the past year studies were initiated to ascertain differences in the antibacterial defenses of malnourished and normal newborn infants. Essentially this project is designed to investigate the metabolism and function of neutrophils in cord blood from infants born in the villages of Sta. Maria Cauque and Sto. Domingo Zenocoj in the highlands of Guatemala. From January 18 through March 26 there were 35 births in the two villages under study. Seventeen cord bloods were successfully studied in the field with both metabolic and bactericidal assays.

Serum has been stored for later assay of opsonic activity and immunoglobulin levels. Six of these newborns were of low birth weight (<2500g), eight were of intermediate weight (2500-3000g) and three of adequate birth weight (>3000g). Greater than 99% killing of the inoculum of bacteria (_S. aureaus_ and _E. coli_) was found in 14 samples, with significantly more antibacterial activity in bloods obtained from the adequate birth weight group. Additionally, one sample from each of the low, intermediate, and adequate birth weight groups was very abnormal in that late (3 hour) multiplication of organisms occurred. Further work is in progress to distinguish between a cellular and a humoral (opsonic) defect. These preliminary data suggest that low birthweight may be associated with decreased antibacterial function of whole cord blood, and that, in addition, some infants may manifest a more severe abnormality independent of birth weight.

Highly significant and relevant information continues to be derived from the contract with the International Rice Research Institute, Los Banos, Philippines. During earlier work in this contract new high-protein varieties of rice had been developed at the International Rice Research Institute. The present and ongoing thrust of the contract involves the nutritional testing of these new varieties which includes laboratory analysis followed by biologic testing in animals and man. The essential verification of the nutritional advantage of replacing rice with the conventional (deficient) protein content with the new high-protein rice lies in its actual application in rice-based weaning foods for actively growing pre-school children. In order to establish meaningfully the true nutritional value of the new rice it must be tried in the environment and the specific population for whom this new rice has been bred. Thus, feeding trials involve young Filipino children who have previously subsisted on a diet consisting primarily of ordinary rice, and similarly, in young South Indian children with a similar dietary background experience.

During the last year, a study of the effect of replacing rice of average protein content with rice of higher protein content in a rice-fish weaning diet on nitrogen retention on Filipino male children, 1.5 to 2 years old, was completed. The mean daily nitrogen intakes were 187, 231, and 254 mg/kg body weight for the three diets: low protein rice, intermediate protein rice, and high protein rice. The composite diets had mean amino acid score based on the lysine content of 99, 91, and 85%. The percent nitrogen absorption and retention by the children were similar for the three diets. Thus, nitrogen retention in milligram per kilogram of body weight increased progressively with an increase in the protein content of rice in the rice-fish diet.

During FY 75 Program staff and members of the U.S. Malnutrition Panel have planned a series of mini-workshops to be devoted to the following priority areas of the Malnutrition Panel:

1) "Investigation of the interaction of malnutrition and infection with special emphasis on the mechanisms responsible."

2) "Studies of the health significance and methods of prevention of nutritional anemias."

3) "Influence of environment and host factors on nutritional requirements for health in Asian Countries."

4) "Determination of the effects of malnutrition on mental development, learning, behavior, physical capability and performance."

It is anticipated a position or working paper will be forthcoming from each of these workshops sponsored by the GMB. These working papers will be further evaluated and definitive guidelines and specific recommendations will be formulated for highly relevant research projects to be advertised and funded by either the grant or research contract mechanism.

5. PARASITIC DISEASES

Research in the Parasitic Diseases Program is confined to two parasitic infections of very great importance in Asian countries - schistosomiasis and filariasis.

Schistosomiasis

A major breakthrough in the development of *S. mansoni* of culture systems for studying the growth and development of schistosome parasites was achieved when one of our contractors established a cell line from embryos of the snail host, *Biomphalaria glabrata*. Suitable media and culturing techniques have been described in detail and are being utilized by investigators in studying larval stages of *S. mansoni in vitro*.

Studies of the possibility of biocontrol of schistosomiasis have shown that *Biomphalaria glabrata* snails have the potential of developing acquired specific resistance to trematode infections. This finding will make possible the study of the basic principles of snail immunity.

A major granuloma-inducing antigen has been isolated to a single band on polyacrylamide gel electrophoresis. A radio-immuno assay has been developed using this antigen and the development of antibody against it correlates with the waning of granulomatous hypersensitivity.

Other studies on the mechanisms of immune response have established a connection between cell mediated immunity to schistosome egg antigen and eosinophil behavior *via* the lymphokine, Eosinophil Stimulation Promoter (ESP). The cell type responsible for the production of ESP is the T lymphocyte.

Evidence has been obtained that within the thioxanthene class of schistosomicidal agents, suitable chemical modifications can maintain or even enhance chemotherapeutic activities while markedly reducing acute and long-term (carcinogenic and mutagenic) toxic effects. One of these compounds not only produced parasitological cures after a single oral dose to mice and hamsters infected with *S. mansoni*, but was found to have prophylactic properties.

Filariasis

Studies continued on the development and evaluation of model systems using non-human primates and rodents as hosts for lymphatic-dwelling filarial worms belonging to the genera *Wuchereria* and *Brugia*. Four species of monkeys were inoculated with a Tongan sub-periodic strain of *Wuchereria bancrofti* resulting in developing worms. Thus it has now been definitely established that *W.* bancrofti, thought to be an exclusively human parasite, will mature in monkey hosts. This finding should expand the scope of filariasis research to include a wide variety of studies in this important human disease.

Studies of the physiology and basic biochemistry of filarial parasites have just recently been undertaken. Such basic knowledge is fundamental to an understanding of the filaria-host relationship and to the rational development of improved chemotherapeutic agents. Biochemical studies have included comparison of the metabolism of several genera of filarial parasites. Microfilariae of D. *witei*, *L. carinii*, and B. *pahangi* lose motility in the absence of air. This indicates that chemotherapeutic agents inhibitory to aerobic metabolism would cause these parasites to lose motility and that, possibly, this loss of motility would allow destruction of the parasites by host defenses. In a study of the mode of action of trivalent antimonial compounds, it was shown that the parasite's phosphofructokinase activity is inhibited, whereas, the host enzyme is not affected. Studies of the mode of action of diethylcarbamazine (DEC) indicate an *in vitro* "immune" cell-worm-DEC reaction which results in a clumping respones.

Ultrastructural studies of the development of *Brugia malayi* larvae in the vector's flight muscles have demonstrated a specialized surface which is suggestive of absorptive function during a phase of rapid growth and development. This is the first demonstration of such a function in filarial larvae and it is hoped that complementary biochemical studies will follow.

Other biochemical investigations include a study of the effect of filaria infection in the vector mosquito on purine conversion. One hypothesis is that once inside the mosquito host, filarial larvae may secrete a conversion inhibiting substance and by inhibiting a specific purine converting enzyme (xanthine dehydrogenase) ensure the availability to the filarids of utilizable purines. In any case, this is another lead to an understanding of the way in which antifilarial compounds might work. Intestinal synthesis of cholesterol is being measured in patients with chyluria due to filariasis to determine the physiologic effects of this disease.

6. TUBERCULOSIS

In FY 75,the Tuberculosis Program continued to pursue research under each area of special interest outlined in the U.S.-Japan Tuberculosis Program guidelines.

Fractionation and identification of mycobacterial antigens - Fractions from unheated *Mycobacterium tuberculosis* culture filtrate prepared by gradient acrylamide gel electrophoresis (GAGE) and by ion exchange and molecular exclusion chromatography were studied in *in vitro* and *in vivo* test systems. The results of these tests indicated that those fractions which produced greater skin reactivity in homologously sensitized animals than in heterologously sensitized animals were identified with antigens 5, 6, and 7 of the U.S.-Japan reference systems. A similar correlation was found when the fractions were studied to determine their ability to induce blastogenesis in immunologically defined human lymphocytes. To purify this apparent group of species specific fractions, subfractionation techniques and further testing are now being pursued. Strains of mycobacteria from an authenticated, highly characterized, and taxonomically classified culture collection continue to be supplied to qualified scientists in tuberculosis research and related fields. The surveillance of all the cultures in the collection was completed this year. The service providing serotyping materials and the serological classification of the atypical mycobacteria continued. A new serotype and two new strains of *M. szulgai* were identified. An improved antiserum was added to the existing reference system for antigens of *M. tuberculosis*. The kit is distributed widely.

Pathogenesis and immunity in tuberculosis - Two antituberculosis vaccines are currently being studied in animal models: 1) BCG, a living vaccine, and 2) Youmans' mycobacterial ribosomal preparation, a non-living vaccine. In the monkey model the route of administration of BCG vaccine significantly affects the degree of protection against aerosol challenge with virulent tubercle bacilli. Inhalation of the vaccine or administration by the intravenous route confers a higher degree of resistance to challenge than does intracutaneous administration of vaccine. The protection afforded by aerosolized vaccine was found to be related to the total dose of viable units (vu) administered through the highest dose tested, 10^5 vu. As an adjunct to this study, it was found that previously sensitized guinea pigs did not display clinical signs of pulmonary distress after exposure to aerosolized BCG; however, there was an indication of a slight, but statistically significant, increase in total airway resistance following exposure. A study is presently underway to determine the duration of protection afforded by aerosolized BCG as opposed to intracutaneously administered BCG. Animals challenged eight weeks following vaccination have now gone through a 21-week post challenge and at this time 67% of the control animals and 17% of animals in each of the vaccine groups have died with overwhelming disease.

The need for T-lymphocytes in the development of both tuberculin sensitivity and anti-mycobacterial immunity in BCG vaccinated mice was demonstrated using adult thymectomized, irradiated mice. Intravenous inoculation of immunizing doses of BCG into these mice resulted in a persisting systemic bacterial infection which resulted in the death of more than 95% of the mice within 100 days. In the virtual absence of immunocompetent T-lymphocytes in these mice, the ongoing tissue response in the lung eventually caused the death of most of the animals due to pulmonary insufficiency. However, the repeated injection of calf thymosin restored the immunocompetence of the T-cell depleted host.

Migration inhibitory factor (MIF) and a new type of interferon (Type II) are released into the circulation after mice have been sensitized intravenously with viable BCG and challenged later with old tuberculin. In this *in vivo* production of mediators the activity of MIF was at least 100-fold greater than the MIF induced *in vitro*. Two components of BCG, the protein PPD and the lipid P3 which were necessary for the production of MIF, were also necessary for the induction of resistance to a virulent aerosol challenge. In preliminary studies, the prodction of MIF *in vivo* in mice seems to be associated with both B and T-lymphocytes. Other studies involving the basic immune mechanisms of the mycobacterioses continue.

This year a task group convened at the National Institutes of Health to discuss the role of soluble mediators of cellular immunity in tuberculosis. These substances have been shown to be of immense biological and clinical importance and are the subject of wide-spread investigation. A general overview of the problem area was discussed, those areas where the U.S.-Japan Program could provide support were identified, and a list of priorities to facilitate orderly progress of the research were developed.

7. VIRAL DISEASES

Research in the Viral Diseases Program is limited to studies on rabies and on arboviruses with emphasis on dengue and the pathogenesis of hemorrhagic fever.

Rabies

It was established that protection from virulent intracerebral challenge with rabies virus is directly dependent on the level of specific neutralizing or lytic antibody present at the time of challenge. Several newly developed evaluation techniques, plaque reduction, immunolysis and complement fixation, were compared with the mouse inoculation technique and were found to be less costly and time consuming and equally accurate. The Rabies Fluorescent Focus Inhibition Test (RFFIT), was also shown by other workers to be more reproducible than the mouse neutralization test, requiring less time for completion and is less expensive. Inactivation of rabies virus by beta-propiolactone was evaluated by the RFFIT and shown to leave no detectable residual infective virus.

Attempts at reproducing and explaining the "early death" phenomenon among vaccinated animals are underway. In a sequential manner the role of antibody, immune cells and killed and live antigens are being examined. The development of antibody and variation in histopathology will be studied.

A major rabies glycoprotein has been extracted by exposure to alkaline EDTA. Physical chemical procedures attest to the purity of the glycoprotein preparation. This protein consists of a single polypeptide chain with a molecular weight of 70,000 dlatons. Its biological activity was demonstrated by immunization of hamsters, rabbits and mice. Studies in this area will be expanded.

Dengue

Recent studies on skin biopsy material from dengue patients indicate that skin rashes in these cases are likely to be immunopathologic in nature. It also has been shown that in acute stages of Dengue Hemorrhagic Fever and Dengue Shock Syndrome, B lymphocytes contain dengue antigen-antibody complex on cell surfaces in the form of an immune complex.

Further studies on the role of complement in dengue hemorrhagic shock have indicated that the shock syndrome in dengue hemorrhagic fever might be caused by an extensive complement activation due to the occurrence of large amounts of immune complexes in circulation.

In studies of the epidemiology of dengue virus in the jungle of Malaysia it has been shown that dengue is a zoonosis involving wild monkeys in the primary rain-forest. Recent emphasis has been on attempts to identify the mosquito species which serves as the important jungle vector. Evidence has been obtained showing that enzootic dengue, involving at least three of the four known virus types, occurs in the high canopy of isolated primary rain-forest and that Aedes 'niveus' mosquitoes are most likely the jungle vectors.

Vector studies have uncovered a marked variation in susceptibility to dengue viruses and the stability of this character from generation to generation. This could be of great importance in controlling epidemic dengue. Results suggest that it might be possible to achieve disease control in an area by replacing a susceptible vector population with a more refractory strain of the same species.

II. Special Concerns on Infectious Diseases - International Centers For Medical Research Program

In response to the expressed desire of the Congress (P.L. 86-610), a program for the International Centers for Medical Research was established in 1960 by the National Institutes of Health "to advance the status of the health sciences in the United States and thereby the health of the American people" by expanding collaborative research between U.S. universities and selected foreign institutions and investigators.

The four grants funded under this Program are awarded for five years with continued support contingent upon program review at the end of each year of operation. One of the most important features of this program is that of long-term support, as contrasted with the transiency which has characterized a number of other international projects. This is a necessary ingredient for the full development of university careers in science which will effectively utilize the international medical experience of individual scientists. A major advantage of this program is that it provides opportunities for established and highly competent investigators to exploit valuable research opportunities abroad without losing their university affiliations and faculty appointments. The long-term support of the ICMR's does not represent a form of foreign aid. Rather the primary objective is a collaborative effort in biomedical research of specific relevance to the health of the American people and to the U.S. scientific community. The four current ICMR grants were renewed for an additional five years in FY 1975.

Purpose and Scope

The ICMR Program has as its principal objective the provision of high quality research and scientific opportunities for Americans in the broad fields of geographic medicine, and in response to the special opportunities existing within each ICMR framework. The length of the research period overseas is a discretionary concern of the ICMR program director, and is obviously determined by both the interests of the prospective investigator and the specific opportunities available within the given ICMR unit. Consistently, the emphasis is upon the quality rather than the quantity or duration of the research programs.

The aggregate ICMR units are serving increasingly as a national resource for utilization by senior as well as less experienced scientists to create a comparatively modest pool of investigators with a sustained career interest in international or geographic biomedical research. In this regard, the ICMR program directors are encouraged to establish a selective, interdisciplinary scientific program. Their activities encourage and accommodate a number of parent university departments other than the department which primarily sponsors the ICMR operation. Particular advantage is taken of special strengths within the overall parent university, including opportunities in health-related fields outside of the classical confines of tropical medicine.

During FY 1971, the NIH initiated a program element within the overall ICMR framework. Designated as the ICMR Research Associate Program (IRAP), this centrally coordinated activity is specially designed to recruit investigators on a national basis and specifically from universities that do not have an ICMR.

As far as is possible, the ICMR core grant is used for research support at the off-shore ICMR site rather than at the parent, domestic university. In this regard it is accepted that some preparation preliminary to an overseas assignment is frequently necessary or desirable, and as many items of equipment and supplies as feasible are purchased in the United States.

Program

The four institutions participating in the ICMR program at present, and their respective areas of research interests are as follows:

1. The Johns Hopkins University ICMR in Baltimore with its overseas site in Dacca, Bangladesh is emphasizing research on diarrheal diseases including cholera and shigellosis, malnutrition, immunology and infection, ecology and animal behavior and the epidemiology of malaria. The Program Director is Dr. Frederik B. Bang.

2. The University of California ICMR under the direction of Dr. Albert Rudnick is based in San Francisco and Kuala Lumpur, Malaysia. Current research interests include arbovirology and entomology, community health (medical anthropology, behavioral epidemiology, transcultural psychiatry, malnutrition and rural health), human genetics and parasitology (emphasizing the biological control of schistosomiasis.

3. The University of Maryland ICMR in Baltimore and Lahore, Pakistan is under the direction of Dr. David Clyde. Research in Lahore is centered around as integrated program on the genetics and field ecology of mosquito vectors. Compound chromosome techniques have been developed and will soon be applied in the field in a pilot program of biological control.

4. The Tulane University in New Orleans has an overseas ICMR base in Cali, Colombia. Dr Paul Beaver is directing a broad research program including clinical, immunological, epidemiological and biochemical aspects of malnutrition, parasitic diseases and mycoses. Other studies include socio-anthropological and demographic aspects of community medicine.

Individual Contract and Agreements Tabulation

U.S.-Japan Cooperative Medical Science Program--Cholera Branch: GMB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY 75 Funding FY 76 Plans
AI-32502	Jefferson Medical College Smith, Harry L., Jr.	Operation and Maintenance of a Cholera Vibrio Reference Laboratory. Makes high quality cholera typing sera and distributes it at no cost to laboratories around the world as well as within the U.S. Maintains a vibrio collection from all known outbreaks of cholera and noncholera vibrio-associated diarrheas. Maintains a record of the characteristics of these cultures for technical and epidemiological comparisons. Essentially a service contract.	$ 22,261 Readvertize
AI-02751	Johns Hopkins University Pierce, Nathaniel	Evaluation of Purified Cholera Toxins and Toxoids as Immunizing Agents Against Cholera. Maintains a laboratory and talent for performing antitoxin titrations in ileal segments in rabbits and also provides the dog model for studying vibrio infections. Essentially a service contract.	$ 80,951 Renewal
AI-02091	Research Foundation State University of New York Craig, John P.	Operation of a Laboratory for Evaluation and Measurement of Cholera Toxins, Antitoxins and Toxoids. Maintains a laboratory and talent capable of performing antitoxin combining power (ATCP) mouse challenge studies and permeability factor (PF) measurements in rabbit skin. Essentially a service contract.	$ 97,205 Renewal

Individual Contract and Agreements Tabulation

U.S.-Japan Cooperative Medical Science Program--Cholera   Branch: GMB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY 75 Funding FY 76 Plans
AI-02101	The University of Texas Medical Branch at Galveston Peterson, Johnny	Study of Cholera Toxoids in Human Volunteers. Maintains a prison volunteer capability to testing the ability of cholera immunogens to produce circulating antibody and/or antitoxins. Essentially a service contract.	$ 71,930 Renewal
AI-32514	Wyeth Laboratories, Inc. Rubin, Benjamin	Production and Purification of Cholera Toxin and Vaccine (Toxoid) Production. Manufacturing contract.	$ - 0 - Terminated
AI-42549	University of Kentucky Benenson, Abram	Study of Incidence of Diarrhea Caused by Enterotoxin-producing *E. coli*. Epidemiological surveillance contract for obtaining the incidence and importance of enterotoxin in diarrheas in various populations.	$ 53,735 Renewal
AI-42548	University of Virginia Guerrant, Richard	Study of Incidence of Diarrhea Caused by Enterotoxin-producing *E. coli*. Epidemiological surveillance contract for obtaining the incidence and importance of enterotoxin in diarrheas in various populations.	$ 66,386 Renewal

Individual Contract and Agreements Tabulation

U.S.-Japan Cooperative Medical Science Program -- Environmental Mutagenesis and Carcinogenesis

Branch: GMB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Funding* FY76 Plans
	Brookhaven National Laboratory Dr. Arnold H. Sparrow	To determine the effects of environmental mutagenesis utilizing higher plants as test systems. Work is continuing as scheduled.	$168,000 Renewal
	Oakridge National Laboratories Dr. Jerry Ulrickson	To continue to catalog new data and to update the Environmental Mutagen Information Center (EMIC).	$122,500 Renewal
	University of Wisconsin Dr. Robert DeMars	To study mutagenesis using cultured human cells. The results of this work will be forthcoming in FY76.	$87,000 Renewal
	University of Washington Dr. Peter Nute	To develop methods to detect point mutations in somatic cells. Work is progressing as scheduled.	$58,000 Renewal
	Jackson Laboratory Dr. Thomas Roderick	To develop a detection system for recessive lethals. Work is continuing and results will be available in FY76.	$50,000 Renewal
	Jackson Laboratory Dr. Thomas Roderick	To develop new mouse strains to maximize sensitivity of a point mutation assay. Work is progressing as scheduled.	$33,845 Renewal
	National Institute of Environmental Health Sciences Dr. Frederick de Serres	To develop and improve genetic tests to characterize specific locust mutants in Neurospora crassa. Work has just been initiated.	$68,804 New

*Supported by NIEHS

Individual Contract and Agreements Tabulation

U.S.-Japan Cooperative Medical Science Program--Leprosy Branch: GMB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY 75 Funding FY 76 Plans
AI-22075	University of Hawaii Worth, Robert	Collection and analysis of data on leprosy in the Pingelapese population. This study will not be completed until 1980. It is anticipated the prophylactic effect of DADDS therapy and the required threshold dose of this drug to interrupt the transmission of leprosy will be ascertained.	$ 7,785 Renewal
AI-02283	Stanford Research Inst. Peters, John	Study the metabolism of rifampin (RFM) in leprosy patients receiving this drug or DADDS for therapy. Using thin layer chromatography (TLC) for separation, RFM and its derivatives shows RFM is strongly bound to both polyamide and silica absorbants.	$ 76,000 Readvertise
AI-52500	Leonard Wood Memorial Reich, Claude	Conduct clinical trials of candidate chemotherapeutic compounds in leprosy patients in Cebu, Philippians. Long term response to various drug regimens-rifampin plus dapsone and rifampin plus DADDS are evaluated in lepromatous patients. Rifampin continues to be bactericidal and reduces the BI (bacterial index) more rapidly than DDS or DADDS.	$149,993 Renewal
AI-42519	Stanford Research Inst. DeGraw, Joe	Produce 10 gms of antileprotic material (2,4-diamino-5-methyl-6-alkyl quinazoline)	$ 3,267 Completed

Individual Contract and Agreements Tabulation

U.S.-Japan Cooperative Medical Science Program--Leprosy Branch: GMB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY 75 Funding FY 76 Plans
AI-42556	University of Illinois Guttman, Helene	Studies on the cultivation of *M. lepraemurium*. Fetal calf serum appears to be a better supplement to Nakamura's basal medium than goat serum.	$102,974 (24 months)
AI-52500	Univ. of California Spitler, Lynn	Preparation of transfer factor (TF) from Dharmandra antigen positive and negative normal volunteers; studies on ability to passively transfer *in vivo* and *in vitro* correlates of hypersensitivity are in progress.	$111,500 -0-
2-Y01-AI-10004	U.S.P.H.S. Hospital, San Francisco Levy, Louis	Microbiological studies on leprosy skin biopsy specimens. The studies on drugs with activity against *M. leprae* in the mouse footpad model continues.	$ 15,000 $ 30,000
2-Y01-AI-10004-02	U.S.P.H.S. - CDC, Atlanta Shepard, Charles	Continue to investigate the activity of dapsone and other anti-leprotic drugs using a pharmacokinetic method in the mouse footpad model. Drug combinations are currently being evaluated for DDS-resistant infections.	$ 86,000 $ 87,000

Individual Contract and Agreements Tabulation

U.S.-Japan Cooperative Medical Science Program-- Malnutrition Branch: GMB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY 75 FY 76	Funding Plans
HD-70726	International Rice Inst. Juliana, B.	Field studies to evaluate rice with high protein content in Filipino children - in progress	$ 12,430	Renewal
HD-42859	Pan. American Health Org. Katz, Michael	To conduct studies on the interaction between malnutrition and infection - in progress	$101,665	Renewal
HD-22737	Pan. American Health Org. Mata, Leonardo	Analysis of data from an ecological study of infection of malnutrition and growth of children in a Guatmalan Indian Village. Dr. Mata is now completing a monograph detailing the characteristics of the village, methods of investigations and the extensive conclusions derived from detailed analysis of the data. It is anticipated the monograph will be completed by summer, 1975.	-0-	Terminate
HD-42816	St. Joseph Hospital Coursin, David	Instrumentation design for electrophysiological studies. The portable electronic aquisition system is now in the operational prototype stage, capable of producing programmed pattern stimuli and recording two channels of electrophysiological data along with three event monitors.	-0-	Renewal
NIH- C-374	National Academy of Sciences Katz, Michael Keusch, Gerald	Conference support - The latent effects of malnutrition and infection during pregnancy as determinants of growth and development of the child. Proceedings to be published.	-0-	Terminate

Individual Contract and Agreements Tabulation

U.S.-Japan Cooperative Medical Science Program--Parasitic Diseases Branch: GMB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY 75 Funding FY 76 Plans
AI-42504	Univ. of Michigan Blankespoor, Harvey	To supply investigators with animals (mammals and snails) infected with various strains of *Schistosoma mansoni*, *S. japonicum* and *S. hematobium*. Approximately 8200 infected mammals and 5300 infected snails were shipped to qualified investigators in 1974.	$113,749 Renewal
AI-90092	Univ. of Georgia McCall, John	To supply investigators with animals and vectors infected with various filarial species. To test various compound for chemotherapeutic and/or chemoprophylactic activity against filarial infections. 128 requests for filariasis research materials were filled for 31 investigators representing 25 institutions. Fifty carefully selected compounds were tested for therapeutic and/or prophylactic activity against filarial infections.	$ 57,961 Renewal
AI-32518	Univ. of California, LA Voge, Marietta	To develop primary cell lines and to establish permanent cell lines derived from tissues of fresh molluscs...The cell line developed under contract #AI-22527 was studied with particular emphasis on chromosome numbers. In addition, miracidia of *S. mansoni* added to cell cultures remained active for 10 days.	Terminated

Individual Contract and Agreements Tabulation

U.S.-Japan Cooperative Medical Science Program--Parasitic Diseases Branch: GMB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY 75 Funding FY 76 Plans
AI-22527	Oregon State University Bayne, Christopher	To develop primary cell lines and to establish permanent cell lines derived from tissues of fresh molluscs...Emphasis was placed on development of techniques of freeze preservation of cells. Such techniques are currently being standardized and refined.	$46,359 5,280 Supplement Terminated
AI-22525	Clinical Pharmacology Research Institute Hansen, Eder	To develop primary cell lines and to establish permanent cell lines derived from tissues of fresh water molluscs...A cell line was established from embryos of Biomphalaria glabrata. Suitable media and culturing techniques have been described in detail and are being utilized by other investigators in studying both parasite and host.	Terminated

Individual Contract and Agreements Tabulation

U.S.-Japan Cooperative Medical Science Program--Tuberculosis   Branch: GMB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Funding FY76 Plans
AI-80640	Trudeau Institute Dr. Maurice J. Lefford	Authentication, maintenance, and distribution of a mycobacterial culture collection. To supply investigators throughout the world with cultures of biochemically characterized and virulence tested mycobacteria. 775 cultures were distributed. The collection now consists of 159 strains. Surveillance of all the cultures was completed this year.	$53,757 Readvertised
AI-02079	National Jewish Hospital Dr. Werner B. Schaefer	To serve as a reference laboratory for the identification of unknown strains of mycobacteria and to provide standard sets of antisera suitable for serotyping certain groups of mycobacteria together with the corresponding reference strains. 352 reference reagents (strains and antisera for serotypes) were distributed. A 28th serotype of the *M. avium-intracellulare* complex, represented by 4 strains, was identified and included in the collection.	$24,164 Indefinite
AI-22521	Hazleton Laboratories Dr. Robert C. Good	To study the duration of protection of BCG aerosol vaccination in monkeys. Aerosol BCG vaccination has proved highly effective in the monkey model and has been shown to be dose dependent. Work is progressing as scheduled on the "duration" experiment.	$149,860 Work completed

Individual Contract and Agreements Tabulation

U.S.-Japan Cooperative Medical Science Program--Tuberculosis Branch: GMB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Funding FY76 Plans
AI-52501	Northwestern University Dr. Guy Youmans	To prepare a mycobacterial ribosomal vaccine and monitor the inoculation of it in guinea pigs. The vaccine was prepared, tested in mice prior and post inoculation of the guinea pigs. The vaccine was judged to be potent. The results of the guinea pig experiment will be forthcoming in FY76.	$10,000 Completed FY76
AI-52508	Medical Research Associates, Inc. Dr. Robert A. Goldstein	To test mycobacterial fractions of culture filtrate (CF) by lymphocyte blastogenesis. The CF fractions were tested using lymphocytes from tuberculin positive and negative persons. The results correlated well with the results of skin testing the fractions in guinea pigs. The fractions having the highest activity were identified with bands 5, 6, and 7 of the U.S.-Japan reference system.	$4,500 Completed
AI-52522	Medical Research Associates, Inc. Dr. Robert A. Goldstein	To test mycobacterial fractions of culture filtrate and cell extract by lymphocyte blastogenesis. This work is progressing as scheduled and results will be forthcoming in FY76. The results will be compared with the testing of fractions in guinea pigs and by electroimmunophoresis.	$34,300 Completed FY76

Individual Contract and Agreements Tabulation

U.S.-Japan Cooperative Medical Science Program--Tuberculosis Branch: GMB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY75 Funding FY76 Plans
AI-42555	Hazleton Laboratories Dr. Robert C. Good	Evaluation of Youmans' ribosomal vaccine in guinea pigs. Preliminary data indicates a protective effect which is dose dependent. The final evaluation and assessment of this vaccine will be completed in FY76.	$44,932 Work Completed
AI-32503	Eastern Virginia Medical School Dr. George L. Wright	To fractionate and characterize mycobacterial antigens by gradient acrylamide gel electrophoresis (GAGE). Of the culture filtrate fractions prepared and studied this year, 15 were selected for subfractionation. Fractions from cell extract will soon be available for detailed immunological testing.	$35,010 Renewal
AI-42513	Case Western Reserve Dr. Thomas M. Daniel	To establish the nomenclature of mycobacterial cell extract reference materials. The relationship to the original 001 reference kit materials was determined. An improved CF antiserum (002A) was produced and is available for distribution.	$3,633 Work completed in FY75
AI-42525	Case Western Reserve Dr. Thomas M. Daniel	To test serologically mycobacterial fractions. Culture filtrate fractions prepared by program were analyzed by microimmunodiffusion and immunoelectrophoresis. Prints of the patterns were made available for planning of subfractionation. Cell extract fractions and culture filtrate subfractions are now being studied.	$28,585 Completed FY76

Individual Contract and Agreements Tabulation

U.S.-Japan Cooperative Medical Science Program--Viral Diseases Branch: GMB

Contract Number	Contractor Principal Investigator	Objectives and Findings	FY 75 Funding FY 76 Plans
AI-12199	Michigan Department of Public Health Mitchell, John	To develop an improved method for inactivation of rabies virus without significant loss of immunogenicty...18 10 liter lots of rabies vaccine were inactivated with the betapropiolactone procedure developed under this contract. No residual infective rabies virus was detected. This inactivated vaccine was shown to protect guinea pigs when administered within 24 hours of intramuscular challenge with rabies street virus.	$57,030 $58,990
AI-12204	University of Michigan Eckert, Edward	To isolate and characterize protective antigens of rabies virus...The major rabies glycoprotein has been extracted by exposure to alkaline EDTA. Physicochemical procedures attest to the purity of the glycoprotein preparation and its chemical characteristics have been defined. The biological activity of the glycoprotein preparations have not been fully explored.	Terminated Readvertised
AI-12292	Wistar Institute Wiktor, Tadeusz	To develop simple and economical procedures for the evaluation of the protective activity of rabies vaccines...It was shown that protection from intracerebral challenge with rabies virus is directly dependent on the level of specific neutralizing or lytic antibody present at the time of challenge. An adapted guinea pig potency test has been shown to be an effective means of assessing protective activity.	Terminated Readvertised

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PART B - STAFF PUBLICATIONS

None

Research Conferences and Workshops

In addition to program elements involving research grants and contracts, the U.S.-Japan Cooperative Medical Science Program includes a provision for regular scientific conferences and workshops. These include joint meetings with Japanese counterpart scientists as well as special conferences for American investigators working in the scientific areas of special interest to a given Panel. These workshops and conferences serve to stimulate interest and enthusiasm which leads to the development of new scientific approaches to the seven disease categories within the Program, and they also facilitate the timely exchange of research data, methods and protocols. The Joint U.S. and Japanese Panels meet annually, alternating between locations in the United States and Japan. A notation of conferences and workshops sponsored during FY 1975 follows:

Meeting Title and Location	Sponsoring Panel
August 5-7, 1974 Joint Environmental Conference Tokyo, Japan	Environmental

Case presentations, laboratory testing, human monitoring, and strategy for the protection of the human genome were agenda items.

Meeting Title and Location	Sponsoring Panel
August 5-7, 1974 Joint Viral Diseases Conference Tokyo, Japan	Viral Diseases

Emphasis was placed on studies of arboviruses, hemorrhagic fever and rabies.

August 8-9, 1974
Joint Committee Meeting
Tokyo, Japan

The Joint Committee composed of the U.S. Delegation and the Japan Delegation, heard and discussed the scientific reports of the U.S. and Japanese Panel Chairmen. Also, the Committee accepted the recommendations of the review group that the Viral Diseases and Malnutrition Panels be continued. Plans for a Second Five Year Report were formulated.

Meeting Title and Location	Sponsoring Panel
August 17-19, 1974 Joint Parasitic Diseases Conference Nagasaki, Japan	Parasitic Diseases

A broad spectrum of papers on these two diseases made up the program content.

Meeting Title and Location (con't) Sponsoring Panel

September 25-27, 1974 Tuberculosis
Joint Tuberculosis Conference
Kyoto, Japan

The conference was concerned with the chemistry and biochemistry of mycobacterial constituents, immunity in tuberculosis, tumor regression mediated by BCG or mycobacterial components, and delayed hypersensitivity. Other related subjects were included on the agenda.

October 14-16, 1974 Cholera
Joint Cholera Conference
Kyoto, Japan

Included in this meeting were sessions on cholera enterotoxin, bacterial genetics, biochemistry, immunology and non-cholera enterotoxic diseases.

October 16-18, 1974 Leprosy
Joint Leprosy Conference
Kyoto, Japan

This was the Ninth Joint Conference on leprosy research including presentations of both U.S. and Japanese investigators.

October 28-30, 1974 Malnutrition
Joint Malnutrition Conference
Mt. Fuji, Japan

This symposium sponsored by the Malnutrition Panels, was devoted to the influence of environmental and host factors on nutritional requirements.

November 14-15, 1974 Tuberculosis
Tuberculosis Workshop
NIH, Bethesda, Maryland

A workshop was convened to discuss the role of lymphokine mediators in immunity to tuberculosis and related diseases.

December 4, 1974 Environmental
Environmental Workshop
Honolulu, Hawaii

U.S. and Japanese scientists met to discuss the mutagenicity and carcinogenicity of over 150 compounds.

January 30-31, 1975
Joint Subcommittee Meeting
Honolulu, Hawaii

Representatives from the U.S. and Japanese Delegations along with staff from the State Department and NIH met to make plans for the forthcoming Committee

Meeting Title and Location (con't) — Sponsoring Panel

meeting and to work on the Second Five Year Report.

April 2-4, 1975 — Parasitic Diseases
Parasitic Diseases Workshop
NIH, Bethesda, Maryland

A workshop was held to evaluate the current status and prospective program of studies on animal models of human filariasis.

May 8, 1975 — Leprosy
Transfer Factor Meeting
NIH, Bethesda, Maryland

This conference included finalization of the clinical protocol to be used in the controlled trial to evaluate T.F. in lepromatous leprosy patients.

June 24-25, 1975 — Cholera
Cholera Workshop
NIH, Bethesda, Maryland

This workshop was concerned with the structure and function of enterotoxin molecules, including subunits and enterotoxins other than cholera.

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Summary of Program
July 1, 1974 - June 30, 1975
Office of the Scientific Director

The summaries of individual laboratories of the individual laboratories constituting the Laboratory and Clinical Research Program of the National Institute of Allergy and Infectious Diseases follow this report. Together, they reflect an active, productive research program in allergy, immunology, and infections.

The Office of the Scientific Director is charged with the overall direction and administration of Laboratory and Clinical Research. Since the forced demise of the Middle America Research Unit (MARU) and the Laboratory of Microbiology, it has attached several individual scientists for whom compatible assignments could not be found in the Laboratories. These included Dr. Karl M. Johnson and Dr. Patricia Webb who had remained in the Panama Canal Zone to provide project officer support to the continued MARU operation under contract with the Gorgas Memorial Institute. Research related to this contract is separately reported by the Gorgas in an annual report prepared and sent to the Congress. Dr. Johnson and Dr. Webb have provided major support to the virologic programs reflected in that report, particularly continued work on Bolivian Hemorrhagic Fever and Venezuelan Equine Encephalitis. They also were involved in an expanded Yellow Fever research effort. Both are scheduled to transfer to the Center for Disease Control in September, 1975, when the contract terminates.

Dr. Sanford Stone continues his research on experimental allergic encephalitis and collaborates with a group at Albert Einstein College of Medicine focusing on the differences in the pathogenesis in juvenile and adult guinea pigs. He is also collaborating with investigators of the University of Maryland in studies on the transfer of immunity of tuberculosis by sensitized lymphocytes and with a collaborator from the Bureau of Biologics/FDA in the mechanisms of contact sensitization by simple chemicals.

Dr. Roy Repaske has continued his studies on the regulation of growth of various microorganiams by carbon dioxide (bicarbonate). Progress has been slow during the year because the necessity for transfer to new space with extensive renovation interrupted his work for most of the year.

The Board of Scientific Counselors met twice during the year. The fall meeting was the second of two meetings between the Board and all the Laboratory Chiefs on the overall objectives and content of the intramural program. These discussions had been far-ranging, involving a serious examination of the strengths and weaknesses of various areas of research, the determinants affecting program decisions, the role of the Board in the peer group assessment and needs for additional peer evaluation, and the respective roles of the Scientific Director, the Laboratory Chief, and the scientist in the selection and conduct of the research which collectively makes up the intramural program. The Board's summary evaluation, available in separate minutes, was that the programs existent were of high quality, suitable to the intramural rather than extramural programs of NIAID, and were providing valuable information and training potential to the overall biomedical research unit. A weakness both in the NIAID and the NIH as a whole was the extent and depth of research in bacterial diseases and the related organisms. Additional efforts should be made to obtain peer evaluation of individual scientists with this being a vital need before tenured appointments are offered. The Board did not believe it should be used as a vehicle for the peer evaluation process but should continue to be used in an advisory capacit on program.

The spring meeting was devoted to a review of the Associate Program, presentations being made by Associates from each Laboratory. The Board was impressed with the quality of the Associates and their accomplishments during their relatively short training period. It also had valuable suggestions as to how high quality Associates could continue to be attracted in the future under the changed circumstances which apply.

In the administrative area, the Laboratory and Clinical Research Program suffered another reduction in staffing by a total of 14 positions. This reduction, which began in the spring of 1968, now totals 155 positions.

Progress was made on the consolidation of all animal rooms and animal care in the Office of the Scientific Director. With the assistance and advice of the Animal Care Committee, progress was made in bringing animal rooms and caging towards the legal standards required. Training programs were also initiated for animal caretakers.

Project No. Z01-AI-00131-08-OSD

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on Antibody Production and Mechanisms of Hypersensitivity in Histocompatible Inbred Guinea Pigs

Previous Serial Number: NIAID-152

Principal Investigator: Sanford H. Stone

Other Investigators: None

Cooperating Units:
1. Dr. Cedric Raine
   Division of Neuropathology
   Albert Einstein College of Medicine
   New York, New York

2. Dr. Gardner Middlebrook
   Dr. Zulema Reggiardo
   Division of Experimental Pathology
   University of Maryland School of Medicine
   Batimore, Maryland

3. Dr. Harold Baer
   Bureau of Biologics
   Bethesda, Maryland

Man Years:	NIAID Lab Staff	Other	Guest Workers	Total
Total:	24/12			24/12
Professional:	12/12			12/12
Other:	12/12			12/12

Project Description:

For some years we have been using the unique tool of inbred guinea pigs available at the NIH for investigation of particular areas of the immune response concerned with hypersensitivity and protection against infectious disease. Advantages lie in the ability to perform viable adoptive transfers of lymphoid cells and in the capacity to study genetic factors in the hypersensitivity phenomena. For these reasons, we have concentrated recently on (1) transfers of protection against tuberculosis with lymphoid cells and attempts to elucidate the immunological basis for this protection (with Reggiardo and Middlebrook); (2) pathogenesis of an age-dependent, strain-dependent chronic autoimmune encephalomyelitis (CAE) which is strongly reminiscent of multiple sclerosis (MS) in man (with Raine); (3) mechanisms involved in contact dermatitis to oxazolone (with Baer); (4) mechanisms of immune deviation. In recipients protected against tuberculosis which we can transfer with lymphoid cells from BCG immunized donors and which is present in the absence of demonstrable delayed hypersensitivity, (DH) the lack of DH is not due to the presence of suppressor cells in the transferred cell suspension itself. This was established by transferring sensitized spleen cells into previously DH sensitized inbred recipients which remained reactive. After many difficulties with adoptive transfers from juvenile donors which would normally develop CAE (adult donors always transfer a classic acute EAE) we have finally succeeded in routinely transferring EAE to juvenile and adult recipients from juvenile donors. Adult recipients regularly develop acute EAE while juvenile recipients show a somewhat different clinical course, the aspects of which are currently under histological and ultrastructural investigations.

In the oxazolone contact dermatitis model, by comparing adoptive (cells) with passive (serum) transfer models and correlating times of reactivity, it has been determined that a serum antibody component plays a role in the contact reactivity to oxazolone. What type of antibody and how general is this phenomenon are questions currently being considered in this collaboration with Baer's group at BOB. Mechanisms of contact sensitization are of importance, since this group of reactions to simple chemicals has been heretofore approached from a basic and clinical point of view as purely cellular manifestations of an immune response.

Keyword Descriptors: Tuberculosis (immunity), multiple sclerosis etiology contact dermatitis mechanisms, chronic demyelination, genetics of immune response, adoptive immunity, histocompatible guinea pigs, immune deviation, age dependence of autoimmune disease.

Honors and Awards:

Professorial lecturer in Immunology, Howard University Graduate School

Invited lecturer - Gordon Conferences 1974: "Adjuvants"

President and Past-President - NIAID Assembly of Scientists

Publications:

Raine, C. S., Snyder, D. H., Valsamis, M.P. and Stone, S. H.: Chronic experimental allergic encephalomyelitis in inbred guinea pigs. An ultrastructural study. Lab. Invest. 31: 369-380, 1974.

Reiss, E., Mitchell, W. D., Stone, S. H., and Hasenclever, H. F.: Cellular immune activity of a galactomannan-protein complex from mycelia of Histoplasma capsulatum. Infection and Immunity 10: 802-809, 1974.

Snyder, D. H., Valsamis, M. P., Stone, S. H. , and Raine, C. S.: Progressive demyelination and reparative phenomena in chronic experimental allergic encephalomyelitis. J. Neuropath. Exp. Neurol. 34: 209-221, 1975.

Project No. Z01-AI-00132-08-OSD

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Growth Regulation in Microorganisms and in Tissue Culture

Previous Serial Number: NIAID-61

Principal Investigator: Roy Repaske

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Staff	Other	Guest Workers	Total
Total:	21/12			21/12
Professional:	12/12			12/12
Other:	9/12			9/12

Project Description:

A constant characteristic of growth of microorganisms and tissue cultures is an initial lag period or "phase of adjustment" which is formally recognized as a physiological growth phase. This phase has been the subject of much research because it represents a period of time during which "old" cells become rejuvenated and conditions are established for cells to begin exponential growth. Therefore, it was of considerable interest when we found that physiologically old cells of Hydrogenomonas entropha could grow immediately at the maximum exponential rate in response to a critical concentration of bicarbonate ion. This unique response warranted extending the investigation to other organisms to determine if it was a general phenomenon. Microorganisms representative of extremes in nutritional types were selected for these studies (H. entropha, Streptococcus sanguis, E. coli grown aerobically and anaerobically, and Bacillus megaterium). "Old" cells of all organisms tested grew immediately when the appropriate bicarbonate or CO_2 concentration was provided. Strict aerobes responded to bicarbonate ion; facultative anaerobes and anerobes responded to CO_2.

The effect of CO_2 (bicarbonate) is even more pervasive than is suggested above. It also functions as a fundamental regulator of growth. Growth can be started or stopped at will by supplying or deleting CO_2 (bicarbonate), and the rate of growth can be predictably selected by limiting the concentration of CO_2 (bicarbonate). The site of action of CO_2 for growth regulation does not seem to be at any known biochemical reaction by which CO_2 is incorporated into cellular material. Preliminary evidence suggests CO_2 regulates growth by regulating fatty acid biosynthesis. Cell lipids are primarily found in cell

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membranes and membrane biosynthesis has been found to be inextrically associat with cell growth, cell division, initiation of DNA replication and separation of the replicated strands of DNA.

We intend to pursue the preliminary observations and confirm the identity of the CO_2 target system involved with growth regulation. In addition, we will extend CO_2 regulation studies to tissue cultures of normal and transformed cells. Growth idiosyncracies of tissue cultures are analogous to bacterial idiosyncracies which respond to CO_2. Comparisons of normal and transformed cells' CO_2 growth regulating systems may provide a new approach to the study of rapid growth by transformed cells.

Keyword Descriptors: Regulation of Bacterial Growth; Growth Regulation by CO_2; Regulation of Tissue Culture Growth; CO_2 and Tissue Cultures; CO_2 and Lag Period; Lipid Biosynthesis and Growth

Honors and Awards:

Nominated for Chairman, Interassembly Council, Assembly of Scientists

Publications: None

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LABORATORY OF CLINICAL INVESTIGATION

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Z01 AI 00059-02 - The Biology of Human and Animal Parvoviruses 88

Summary of Program
Laboratory of Clinical Investigation
July 1, 1974 - June 30, 1975

Sheldon M. Wolff, M. D., Chief of Laboratory
and Clinical Director, NIAID

Introduction

The year 1974-1975 has been an exceedingly productive one in terms of scientific output and clinical activities. As in recent years, despite obvious problems in staffing, the quality and quantity of work has increased.

Research Accomplishments

IMMUNOLOGIC RECONSTITUTION

The studies of chronic candidiasis as a model of defective cell-mediated immunity have been extended. The prediction that clinical and immunological characterizations would serve to identify various classes of patients has been borne out. Furthermore, these studies have facilitated selection of treatment schedules. One mode of therapy, restoration of cell-mediated immunity with transfer factor, is apparently beneficial. However, the mechanism of action of transfer factor and the importance of using donors with positive Candida skin tests has not been established. (Kirkpatrick and Smith)

The pathogenesis of the cutaneous inflammatory lesions in chronic candidiasis has been studied. The results indicate that the inflammatory response is initiated by direct activation of complement by the alternate pathway. (Sohnle, Frank and Kirkpatrick)

An active fraction of dialyzable transfer factor has been isolated. It contains a substance that is thought to be hypoxanthine. The relationship of this material to cellular immunity is under study. (Kirkpatrick and Smith)

Transfer factor also increases monocyte cyclic GMP and may thereby have an antigen independent effect on immunologic inflammation. (Sandler [Lab. of Cell. Metab., NHLI, NIH], Manganiello [Lab. of Cell. Metab., NHLI, NIH], Smith and Kirkpatrick)

ALLERGIC DISEASES

The Allergic Diseases Section studies the mediators and biochemical pathways which underlie allergic reactions. Histamine has been shown to be selectively chemotactic for human eosinophils between 30 and 200 ng/ml histamine. This is most readily demonstrable when the percentage of eosinophils exceeds 30. At this dose range, preincubation with histamine deactivates the eosinophil's response to histamine as well as other chemotactic factors such as C5a and neither the eosinophil stimulation nor the deactivation is inhibited by H_1 or H_2 receptor antagonists. Above 200 ng/ml, histamine progressively diminishes eosinophil responsiveness to histamine and other chemotactic factors such as C5a or kallikrein which is reversible by H_2 receptor antagonists but not H_1 receptor antagonists. (Kaplan, Clark)

In *vivo* histamine release has been demonstrated in patients with cold urticaria, cholinergic urticaria, and familial vibratory angioedema. Histamine release with cold exposure is rapid in patients with cold urticaria within two minutes after exposure and peaks at five minutes. Plasma histamine reached above 100 ng/ml in one patient with hypotension. Histamine release in vibratory angioedema is even more rapid and peaks between two and three minutes. Histamine release in cholinergic urticaria is slow, by comparison, and remains elevated for over 30 minutes after exercise challenge. (Kaplan, Gray)

A new factor required for the normal functioning of the Hageman factor dependent pathways has been identified. A patient, Ms. Williams, was found to possess no detectable kininogen; however, besides the expected inability to generate bradykinin, Hageman factor dependent coagulation and fibrinolysis were markedly impaired. A high molecular weight form of kininogen has been identified as the factor which corrects all of the abnormalities of this plasma and precedes the feedback effects of kallikrein and plasmin upon Hageman factor. In studies of the mechanism of Hageman factor dependent fibrinolysis, a new form of human plasminogen has been identified which is approximately half the size of native plasminogen. Its formation is dependent upon cleavage of native plasminogen by one or more enzymes possessing an active site serine. Isolated low molecular weight plasminogen possesses two bands at molecular weight 50,000 and 44,000 upon SDS gel electrophoresis, suggesting cleavage of plasminogen into two parts. Activation to plasmin and incorporation of ^{32}P DFP into this low molecular weight plasmin demonstrated that all of the fibrinolytic activity is contained in the 50,000 molecular weight fragment. (Kaplan, Yecies, Meier)

CELLULAR IMMUNOLOGY

Antigen recognition by immune lymphocytes requires an obligatory uptake of antigen by macrophages. The handling of soluble protein antigens by macrophages is complex, although a major portion of most (approximately 80%) of macrophage-bound radiolabelled antigen is either released or degraded rapidly ($T_{1/2}$ three to six hours). In view of previous studies this degradative pathway likely occurs via fluid-phase (macro) pinocytosis. A second functional pathway is not degradative and may occur via an adherence dependent pathway (micropinocytosis). This latter pool is stable for up to 72 hours with respect to TCA and antibody precipitability and is not present on the surface membrane as judged by binding of specifically purified FAB'$_2$ anti-antigen or by proteolytic enzyme susceptibility. Moreover, anti-antigen does not inhibit T lymphocyte recognition of macrophage bound antigen.

Recognition of macrophage-bound antigen precedes via an initial reversible antigen independent binding of lymphocyte to the macrophage. This event requires active macrophage but not lymphocyte metabolism. This initial antigen-independent binding is followed by a period of antigen-dependent binding and cell division if and only if: 1) both macrophage and lymphocyte share identity of membrane H-linked determinants; and 2) the macrophage carries the appropriate antigen for which the lymphocyte bears an immunospecific receptor. Thus, antigen-independent binding would serve to approximate or focus T or B lymphocytes on macrophages without conveying immunological specificity; a subsequent antigen and histocompatibility dependent physical and functional phase of macrophage-lymphocyte interaction would serve to convey specificity and facilitate regulation of genetically controlled immune responses. Further studies to evaluate this model are in progress. (Rosenthal, Greineder, Lipsky, Ellner, Rosenwasser, Barcinski)

CLINICAL STUDIES OF COMPLEMENT ABNORMALITIES IN MAN

During the past year, major emphasis has been placed on therapeutic studies of hereditary angioedema (HAE). A group of patients is being followed for signs of EACA toxicity and is on long-term therapy with this drug. During the year, a second drug was evaluated as an agent which might be useful in therapy. Provera, high dose progesterone therapy, of HAE proved ineffective in therapy. A third hormonal agent is now under double-blind study. This drug is the impeded androgen, Danazol. A method of approach to the problem of dental surgery of these patients has been developed. Two units of fresh frozen plasma are given on the day prior to major dental work. Six patients underwent seven major dental procedures under this therapy without any attacks of oral swelling. This same group of patients underwent 23 previous procedures with 18 attacks of marked swelling and four life threatening HAE attacks.
(Frank, Jaffe, Gelfand, Atkinson [Washington Univ., St. Louis, Missouri])

PATHOPHYSIOLOGY OF HEMOLYTIC ANEMIA

A series of studies which examine the effect of antibody and complement on the immune clearance of erythrocytes was continued. Erythrocytes coated with IgM antibody are cleared by a complement dependent mechanism by the RES of the liver and then are released back into the circulation as Coombs positive cells. Studies with cold agglutinins demonstrated that this above noted effect is independent of antibody and only depends on the interaction of complement fragments with cells of the RES. Also, the sites of C3 deposition were shown to differ for anti I cold agglutinins and other IgM anti-RBC antibody and were found to operate essentially independently of one another.
(Frank, Jaffe)

STUDIES ON THE INTERACTION OF ANTIBODY AND COMPLEMENT ON THE PRODUCTION OF IMMUNE DAMAGE

The direction of these studies has changed somewhat in the past year, and the effect of various complement fragments on cell function and on host defense mechanisms is the major area under study. In collaboration with Drs. Shearer and Parker of Washington University of St. Louis, we showed that complement may play a major role in the rate of tumor growth. Components through C3 deposited on a tumor cell membrane, via a mechanism which does not cause sufficient damage to injure the cell (too few complement activation sites), causes a marked increase in the rate of tumor cell growth. Components beyond C3 are not required for this effect (Reported in The Journal of Experimental Medicine).

These complement fragments on cell membranes are of great importance in that they appear to bind to specific receptors which can be demonstrated within the tissue sites. In collaboration with investigators in the NCI, a new reclassification of a wide variety of lymphoreticular malignancies has been in progress. More important, within the past year, in collaboration with Drs. I. Green and M. Gelfand, members of the Clinical Immunology Section have demonstrated the presence of a C3b receptor in the human glomerulus. This may explain why complement containing complexes are deposited in this organ leading to the production of renal disease. New and simple methods have also been developed to demonstrate the presence of these receptors in cell suspension and tissue section, which make use of the fact that fluorescein-labelled bacteria can have deposited on their surface C3 via activation of the alternate-complement pathway. These bacteria can be used as a simple reagent to detect C3 receptors. (Frank, Reynolds, Gelfand, Fauci, Green [LI, NIAID, NIH])

OBSERVATIONS OF SECRETORY AND CELLULAR IMMUNITY IN THE RESPIRATORY AND GASTROINTESTINAL TRACTS

The ultrastructure of canine secretory component and J-chain are being investigated now that they have been isolated and purified from secretory IgA; the combination of these chains with 7S IgA monomer may elucidate the assembly of the secretory IgA molecule. (Reynolds, Thompson)

While investigating the origin of precursor cells that differentiate into immunoglobulin secreting plasma lymphocyte cells in the gastrointestinal tract, the interactions of orally administered antigens and processing cells (T-lymphocytes) in gut-associated lymphoid tissue (Peyer's patches) were studied in adult guinea pigs. A detailed identification of the lymphoid cell populations in Peyer's patches and an assessment of lymphocyte activity (afferent and efferent T-cell functions) were made. (Levin [Univ. of Chicago, Chicago, Ill.], Reynolds).

The general area of pulmonary host defenses and nosocomial infections is being investigated, using Pseudomonas pneumonia as the prototype infection because of its frequent occurrence in immunosuppressed patients who are most susceptible to opportunistic microorganisms. Initial work has been done in rabbits to explore ways of manipulating antibody production in the respiratory tract and to assess the effectiveness of these antibodies in promoting phagocytosis and killing by alveolar macrophages. IgG was found to be the principal opsonin in the lung; maximal amounts of IgG opsonic antibody could be produced with a combination of intramuscular and intranasal (local secretory IgG produced) immunization. IgA antibody was induced with intranasal vaccination but was judged to have less opsonic activity. Competent phagocytic cells are essential for the pulmonary defenses. In addition, intranasal immunization with Pseudomonas lipopolysaccharide sensitized respiratory lymphocytes which could be induced to release migration inhibition factor, which is an _in vitro_ correlate of delayed hypersensitivity or cellular immunity. However, evidence of cellular immunity in the coordinated host defense of the lung against Pseudomonas infection needs to be assessed further. Where possible, similar studies have been conducted in human subjects. A Pseudomonas vaccine (Parke-Davis, Inc., IND 185, BOB) has been evaluated in patients considered at high risk to develop Pseudomonas infections such as those with acute hematologic malignancies, cystic fibrosis, and chronic pulmonary diseases. The immunogenicity of the vaccine is good but the protective effects (i.e., humoral agglutinating antibodies against somatic Pseudomonas antigens) were not striking in the small number of subjects available for comparison (about 50). (Kazmierowski, Reynolds, Durbin)

IMMUNOSUPPRESSION

Studies of the identification, characterization, and chemotherapeutic regulation of lymphoid cells in man and experimental animals have demonstrated that various subpopulations of lymphoid cells not only differ markedly in their sensitivity to different chemotherapeutic regimens, but also are quite distinct both in their participation in immunologically mediated diseases, and in their contribution to host defense mechanisms. These studies have also demonstrated that compartmentalization rather than destruction of lymphoid cells is an important mechanism of the suppression of immunological competence seen both in untreated disease states and during chemotherapeutic regimens such as corticosteroids. Corticosteroids cause a redistribution of recirculating lymphocyte subpopulations from the intravascular space to other body compartments including the bone marrow. These observations have lead to an examination and characterization of the lymphoid cell populations in the bone marrow in normal humans, in various disease states, and in experimental animals, which has resulted in clinical relevant information. The bone marrow was formerly thought to be devoid of mature thymus-derived (T) cells. Studies in our laboratory have demonstrated that the bone marrow contains a small fraction (10%) of functionally mature T cells in addition to 20 to 25% bursa equivalent (B) cells, and 70% lymphoid cells without detectable markers. These findings may well account for the frequently encountered graft-versus-host reaction seen with bone marrow transplantation as well as for the T cell functional capacity of bone marrow suspensions noted in disease states, during chemotherapeutic regimens, and in certain animal studies. (Fauci, Hurley, Balow, Hunninghake, Parrillo)

CLINICAL MYCOLOGY

Inoculum was discovered to have a profound effect on immune response to experimental cryptococcosis in the guinea pig. Sublethal inocula induced delayed hypersensitivity reactions, MIF production and lymphocyte proliferative responses to cryptococcal antigen. Slowly lethal infection induced specific anergy, and rapidly lethal infection brought nonspecific anergy. This range of response parallels findings in patients with cryptococcosis and suggests utility of the guinea pig model in probing the immune response to this mycosis. (Bennett)

BASIC STUDIES ON PATHOGENIC FUNGI

Study revealed that sexual spores of Histoplasma capsulatum are infective for mice as asexual spores. Sexual cycle of H. capsulatum was found to be better preserved when cultures were maintained in tissue form rather than saprophytic form on agar media. Three new sterols isolated from mutants of Cryptococcus neoformans and Aspergillus fennelliae resistant to polyene antibiotics were identified.

Definite relationship between the temperature tolerance in vitro and the type of lesions produced by the isolates of Sporothrix schenckii was found. The fixed cutaneous lesions were produced by 35C type while lymphangitic and deep sporotrichosis were caused by 38C type. (Kwon-Chung)

BIOLOGY OF PARVOVIRUSES

Adeno$_2$-SV$_{40}$ mutants provided complete helper functions of adenovirus associated viruses (AAV), indicating that the helper function resides on the retained portion of the adenovirus genome and is not inhibited by the insertion of large fragments of the SV$_{40}$ genome. Infectious DNA extracted from adenovirus 2 potentiated AAV. Replication of a rodent parvovirus, KRV, was inhibited by phosphonoacetic acid. (Dolin,Reichman)

HERPES VIRUS INFECTION

Adenine-arabinoside (ARA-A) treatment of 11 immunosuppressed patients with varicella-zoster infections, as part of a nationwide efficacy trial, appeared to decrease the duration of virus shedding and new vesicle formation. Similar efficacy trials in herpes simplex encephalitis and mucocutaneous herpes simplex infections are currently underway. An in vitro assay system provided a rapid, reliable method to determine ARA-A levels. ARA-A was found to selectively inhibit Epstein-Barr virus replication. Guinea pig models of systemic herpes simplex virus infections were established. (Dolin, Coker, Reichman)

VIRAL GASTROINTESTINAL AND RESPIRATORY TRACT INFECTIONS

Acute viral gastroenteritis induced by Norwalk and Hawaii agents results in a marked, but transient, lymphopenia at the height of illness, which involves all lymphocyte subpopulations (T, B, and null cells). The lymphocytes remaining in the circulation respond normally to mitogens. This lymphopenia may be a result of an acute redistribution of circulating lymphocytes to sites of infection in the gut. Infection with the Hawaii agent is associated with a serum antibody rise to the Hawaii particle as measured by immune electron microscopy, along with a spectrum of clinical illness and histopathologic responses.

Experimental infection in man with Udorn and Georgia strains of influenza virus (H_3N_2) resulted in a mild lymphopenia, with a mild suppression of lymphocyte responsiveness to mitogens. Low levels of specific antigen stimulation were seen in four-week convalescent lymphocyte cultures.

Polymorphonuclear leukocyte function appeared to be normal during influenza, but phagocytosis-stimulated C1-glucose oxidation appeared to be decreased during viral gastroenteritis. (Dolin, Coker, Reichman)

PARASITIC DISEASES

Principal research efforts during the past year were on filariasis, leishmaniasis and toxoplasmosis. Specificity of the IgE response in patients with tropical eosinophilia has been tested in a system of passive sensitization of human basophils to determine whether a specific antigen producing histamine release can be identified. An expedition to the Cook Islands of the Pacific was organized for studies of filariasis, with primary emphasis on immunologic responsiveness of patients with this infection. Patients with leishmaniasis and toxoplasmosis were utilized for tests of cell-mediated immunity and for circulating antigen, respectively. (Neva, Kaplan, Ottesen, Weller [LPD, NIAID, NIH], Heck, Wyler [LPD, NIAID, NIH])

HOST DEFENSE MECHANISMS

Further characterization of human leukocytic pyrogen indicates that the pyrogenic material released by human eosinophils may, in fact, be a different molecule than that released by other circulating blood cells. Thus, leukocytic pyrogen producing cells may each release a different molecular form of pyrogen. (Wolff)

The limulus amebocyte lysate test has been shown to have little clinical utility in the detection of Gram-negative rod bacteremia or septicemia in man. (Elin [CPD, CC, NIH], Wolff)

Human neutrophils contain in their specific granules a material that generates chemotactic activity from serum by activating the complement system. Neutrophils also contain in the neutrophil granules a protease that inactivates the chemotactic factor C5a. Both of these materials are released during phagocytosis but with different kinetics of release, the serum activator being released sooner and with lower phagocytic challenges. (Wright)

PMN's collected by simple venapuncture, NCI-IBM cell separator, and by continuous flow filtration were assayed for phagocytic and bactericidal activity. While cells collected by the cell separator were functionally normal, those collected by adherence to nylon wool in the continuous flow filtration method were found to possess defective phagocytic and bactericidal capacity. These defects were proportional to the length of time the cells were exposed to the nylon filter and the manner in which the cells were eluted from the filter. Similar findings were obtained in measurements of PMN chemotaxis. (Wright)

Administrative, Organizational, Other Changes

We are delighted that this coming year none of our senior staff will be leaving us for the first time in many years. I am also happy to report that Dr. John Gallin will be returning to continue his studies on white blood cell movement and function. Dr. Michael Kaliner will shortly join the Allergic Diseases Section to begin his studies on mediators of the acute allergic response. Sometime during the coming year, Dr. John Atkinson will be returning to join the Clinical Immunology Section. Dr. Atkinson has spent the last year as a Fellow under Dr. Charles Parker in St. Louis, Missouri. Dr. Charles Dinarello will be returning after a year in Boston as Chief Resident in Pediatrics at the Massachusetts General Hospital. Dr. Dinarello will continue his work on the purification of leukocytic pyrogen. Dr. James Balow will remain in the Clinical Physiology Section and continue to provide us with much needed expertise in nephrology. Both Drs. Balow and Dinarello will be supported by the National Cancer Institute as expert consultants. The addition of these five capable investigators to our staff will give us enormous additional strength. Unfortunately, no positions are available to provide these individuals with adequate technical support, and this is a problem that we must come to grips with in the near future.

Honors, Awards and Scientific Recognition

A very large number of honors have come to the members of the LCI. There are far too many to be listed here in their entirety. Dr. Alan Rosenthal was the Chairman of the Ninth Leukocyte Culture Conference and the editor of the book, "Immune Recognition." Dr. Michael Frank is the Chairman of the Complement Workshop and the recipient of a meritorious service medal of the U.S.P.H.S. Dr. Herbert Reynolds is the Chairman of the NIH Clinical Research Committee of the Medical Board. Dr. Charles Kirkpatrick is on the editorial board of the Journal of Allergy and Clinical Immunology; Drs. Rosenthal and Frank serve on the editorial board of the Journal of Immunology; Dr. Wolff serves on the editorial boards of The Journal of Clinical Investigation, the Journal of Infectious Diseases, and American Family Physician; Dr. John Bennett serves on the editorial board of Antimicrobial Agents and Chemotherapy. Many of the members of LCI gave name lectures both here and abroad.

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Project No. Z01 AI 00042-10 LCI
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanisms of Fever and Host Responses

Previous Serial Number: NIAID-12(c)

Principal Investigator: Dr. Sheldon M. Wolff

Other Investigators: Dr. Michael J. Chusid
Dr. Anthony S. Fauci
Dr. Michael M. Frank
Dr. James E. Gadek
Dr. Jeffrey A. Gelfand
Dr. Allen P. Kaplan
Dr. Charles H. Kirkpatrick
Dr. Alan S. Rosenthal
Dr. Daniel G. Wright
Mr. Joseph B. Edelin
Ms. Lois Renfer
Mrs. Kathy A. Ankrom

Cooperating Units: Dr. David W. Alling, OSD, NIAID, NIH (No project no.)
Dr. Warner Barth, Washington Hospital Center, Wash., D. C.
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Dr. Louis Chedid, Pasteur Institute, Paris, France
Dr. Jonathan L. Costa, LCS, NIMH, NIH (No project no.)
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Dr. Charles A. Dinarello, Department of Pediatrics, Massachusetts General Hospital, Boston, Mass.
Dr. Ronald J. Elin, CPD, CC, NIH (No project no.)
Dr. Clement Finch, Department of Hematology, University of Washington Hospital, Seattle, Washington
Dr. John I. Gallin, Department of Medicine, Bellevue Hospital, New York, New York
Dr. Frank H. Gardner, Dept. of Medicine, Presbyterian-Univ. of Pennsylvania Medical Center, Philadelphia, Pa.
Dr. Vee Gill, Microbiology Service, CPD, CC, NIH (No project no.)
Dr. Harvey R. Gralnick, Hematology Service, CPD, CC, NIH (No project no.)
Dr. Geoffrey Herzig, Experimental Hematology Section, POB, MO, DCT, NCI, NIH (Project no.Z01 CM 03422-02 PO)

Dr Ralph E. Johnson, DCBD, NCI, NIH (No project no.)
Dr. Jean C. Kauffmann, Experimental Hematology Section, POB, MO, DCT, NCI, NIH (Project no. Z01 CM 03422-02 PO)
Dr. Arthur S. Levine, DCT, NCI, NIH (Project no. Z01 CM 06830-05 POB)
Dr. George A. Padgett, Washington State University, Pullman, Wash.
Dr. Charles W. Parker, Department of Internal Medicine, Washington Univ. School of Medicine, St. Louis, Mo.
Dr. James B. Sweet, LOM, NIDR, NIH (No project no.)
Dr. Min-Fu Tsan, Division of Hematology, The Johns Hopkins Hospital, Department of Medicine, Baltimore, Md.
Dr. Elliot Vesell, M. S. Hershey Medical Center, Dept. of Pharmacology, Hershey, Pa.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	6-2/12			6-2/12
Professional	3-2/12			3-2/12
Other	3			3

Project Description:

Selected patients are being studied on the wards and in the laboratory. The patients can be divided into the following groups: patients with a) recurrent fever; b) Chediak-Higashi syndrome; c) granulomatous disorders; d) cyclic neutropenia; e) eosinophilia; f) recurrent infections; g) familial Mediterranean fever; h) aplastic anemia; and i) normal volunteers.

Extensive studies are in progress relating to the pathogenesis of fever, granulocyte function, immune and other responses of humans and experimental animals to infections, immunosuppression, etc.

Objectives:

1. To assess varied biologic responses of normal and a variety of patients to pyrogens.
2. To assess the responses of humans to multiple doses of endotoxin (endotoxin tolerance).

3. To intensively study groups of patients with such poorly defined illnesses as familial Mediterranean fever, recurrent fever, Chediak-Higashi syndrome, midline granuloma, Wegener's granulomatosis, cyclic and other neutropenias in an attempt to better delineate the clinical syndrome.

4. To study the mechanisms responsible for the release of endogenous pyrogen.

5. To characterize and purify leukocytic pyrogen.

6. To assess the effect of immunosuppressive agents on human diseases.

7. To explore the pathogenesis of the increased susceptibility to infection in a variety of diseases.

8. To study the effect of fever on drug metabolism.

9. To define the features of the beige mouse which are similar to the Chediak-Higashi syndrome of man.

10. To determine the susceptibility of beige mice to infection.

11. To assess the protective effects of endotoxin and other microbial products in mice challenged with various microorganisms and, if possible, to characterize the humoral and cellular factor(s) responsible for protection.

12. To evaluate the specificity of limulus amebocyte lysate for compounds other than endotoxin.

13. To evaluate the utility of the limulus amebocyte lysate test to detect endotoxemia in man.

14. To identify patients with abnormal neutrophil and/or mononuclear cell chemotaxis and patients with deficiencies in the generation of chemotactic factors.

15. To study the surface charge of human neutrophils after exposure to chemotactic factors.

16. To evaluate the chemotactic properties of products of phagocytosis.

17. To study the effect of *in vivo* endotoxin on the *in vitro* chemotactic activity of human plasma.

18. To identify normal secretory functions of neutrophils.

19. To relate the extracellular release of neutrophil granule products with the generation and inactivation of chemotactic factors.

20. To understand the interactions of neutrophil granule products with circulating monocytes.

21. To study extracellular release of granule products by neutrophils from patients with familial Mediterranean fever (on and off colchicine) and from patients with the syndrome of mucocutaneous candidiasis and multiple endocrinopathies.

22. To purify neutrophil granule products according to selected functions in vitro.

23. To study the functional capacities (chemotaxis, phagocytosis, bacterial killing) of neutrophils collected by continuous flow filtration leukopheresis and by continuous flow centrifugation leukopheresis for transfusion into neutropenic patients.

24. To study the effects of oral and parenteral corticosteroids in the syndrome of cyclic neutropenia.

Methods Employed:

Patients and volunteers receive varying doses of endotoxin and etiocholanolone. Rectal temperatures are taken and a variety of responses are determined: antibody levels, immunoglobulins, plasma cortisol, growth hormone, serum iron, clotting factors, etc.

Microorganisms were grown in appropriate media, quantitated by turbidity and intravenously injected into mice.

Limulus amebocyte lysate was prepared from limulus crabs. The lysate is used to study endotoxemia in man and specificity of the reaction.

Mice are given endotoxin or bacterial products and subsequently challenged at varying time intervals with such infectious agents as Candida albicans, Plasmodium berghei, etc.

Human peripheral leukocytes are incubated overnight with killed S. albus organisms. The resulting supernatant contains leukocytic pyrogen. This material is concentrated by means of alcohol precipitation, dialysis, column chromatography and fractionation, isoelectric focusing and electrophoresis.

Neutrophil chemotaxis is evaluated using a radioassay employing ^{51}Cr-labeled leukocytes and a double micropore filter Boyden chamber previously developed in our laboratory. Mononuclear cell chemotaxis is performed with a morphologic assay. Chemotactic factors are generated by incubating serum with either endotoxins or antigen-antibody complexes (complement related factors) or with kaolin (kinin related factors). The chemotactic factors are then purified by sephadex chromatography.

Phagocytosis is studied using Hypaque-Ficoll purified neutrophils, and latex particles are used for the phagocytic challenge.

Neutrophils and monocytes are separated from peripheral blood of normal volunteers by dextran sedimentation with or without Hypaque-Ficoll separation.

Granules are separated from human neutrophil lysates by differential centrifugation and by ultracentrifugation on sucrose gradients.

Chemotactic factors are generated by incubating serum with endotoxins. Partially purified C5a is prepared by Sephadex chromatography.

Various neutrophil granule enzymes (lysozyme, myeloperoxidase, β-glucuronidase) and non-granular enzymes (lactic dehydrogenase) are measured in leukocyte fractions and in post-phagocytic incubation media.

Neutrophils obtained by leukopheresis (continuous flow filtration and continuous flow centrifugation) are studied for in vitro function or capacities with and without variations in the leukopheresis techniques.

Patient Material:

Patients are admitted to the Clinical Center, as are normal volunteers, for these studies. In addition, patients from other Institutes participate in these studies.

Major Findings:

Human leukocytic pyrogen has been found to be at least two molecules, one from polymorphonuclear leukocytes and the other from monocytes. The pyrogen derived from eosinophiles may represent a third unique molecule.

Cyclophosphamide has produced striking remissions and clinical improvement in patients with Wegener's granulomatosis, nephrosis and a variety of hypersensitivity diseases.

The limulus amebocyte lysate test has limited clinical utility for the detection of endotoxemia or Gram-negative rod septicemia in man.

Neo-WSA, a delipidated material derived from Mycobacteria smegmatis, induces nonspecific resistance to infection in mice and the mechanism differs from endotoxin, the classical compound for the induction of nonspecific resistance.

Human neutrophils contain in their specific granules a material that generates chemotactic activity from serum by activating the complement system. Neutrophils also contain in the neutrophil granules a protease that inactivates the chemotactic factor C5a. Both of these materials are released during phagocytosis but with different kinetics of release, the serum activator being released sooner and with lower phagocytic challenges.

Neutrophils collected by continuous flow filtration leukopheresis are functionally abnormal by *in vitro* assays in direct relation to the time and extent of adherence by the cells to nylon wool filters used by this leukopheresis technique. Pretreatment of leukopheresis donors with corticosteroids protects the neutrophils from the functional changes induced by adherence.

Leukocyte chemotaxis is markedly enhanced by exposing the cells to levamisole *in vitro*. Leukocytes from patients with Job's syndrome variants and Chediak-Higashi syndrome are particularly sensitive to this enhancement effect.

A 13-year-old boy with recurrent pyogenic infections was studied and found to have a previously unrecognized granulocytopathy consisting of abnormal polymorphonuclear bactericidal and chemotactic ability, as well as abnormal *in vivo* skin window responses.

Polymorphonuclear's from four patients with chronic granulomatous disease were found to lack the increase in C-1 glucose oxidation normally seen in cells exposed to PHA, conA, or PWM. These findings inferred a granulocyte membrane defect in the polymorphonuclear's (PMN's) of patients with chronic granulomatous disease.

PMN's collected by simple venapuncture, NCI-IBM cell separator, and by continuous flow filtration were assayed for phagocytic and bactericidal activity. While cells collected by the cell separator were functionally normal, those collected by adherence to nylon wool in the continuous flow filtration method were found to possess defective phagocytic and bactericidal capacity. These defects were proportional to the length of time the cells were exposed to the nylon filter and the manner in which the cells were eluted from the filter. Similar findings were obtained in measurements of PMN chemotaxis.

PMN function before, during and after gastroenteritis viral infection was found to demonstrate decreased glucose oxidation during the acute phase of the illness.

Complement component (C_1, C_4, CH_{50}) levels and opsonic potential of sera obtained before and after heparinization in a group of patients undergoing heparinization were measured. Heparinization had no significant effect upon any of these parameters, suggesting that the *in vitro* effect of heparinization previously described was not correlated with any measurable *in vivo* effects in this group of patients.

Blood cultures, WBC, differential, limulus assay, and NBT slide test were all performed before, during and after dental surgery. Various antiseptic pretreatments were performed. Results obtained in over 90 patients demonstrated that over 80% of patients receiving no local antiseptic treatment developed bacteremia within one hour of the surgical treatment. Pretreatment with a variety of local antiseptics decreased the incidence of bacteremia, with chloramine T reducing the incidence the most (to about 40%).

The platelets of patients with the Chediak-Higashi syndrome have been found to have a markedly reduced number of granules and very low levels of serotonin.

Preliminary studies suggest that etiocholanolone may be a useful agent in the therapy of aplastic and hypoplastic anemia.

Colchicine can be employed as a therapy of the acute attacks of familial Mediterranean fever in addition to its prophylactic value.

Significance to Biomedical Research and the Program of the Institute:

The large number and wide variety of studies listed above provide considerable new knowledge dealing with host response mechanisms to a wide variety of clinical and experimental diseases and the pathogenesis of fever.

Proposed Course:

The above outlined studies are to be continued and some areas are to be expanded.

Keyword Descriptors:

Fever, pyrogens, endotoxins, and host responses.

Publications:

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Project No. Z01 AI 00043-10 LCI
1. LCI
2. Clinical Mycology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Immunology and Chemotherapy of Systemic Mycoses

Previous Serial Number: NIAID-18(c)

Principal Investigator: Dr. John E. Bennett

Other Investigators: Dr. James Balow
Dr. Michael Frank
Dr. Jeffrey Gelfand

Cooperating Units: Dr. David Alling, OSD, NIAID, NIH (No Project No.)
Dr. Charles Meyers, LCP, DCRT, NCI, NIH (No Project No.)
Dr. Robert Diasio, LCP, DCRT, NCI, NIH (No Project No.)
Dr. Sally Harding, Johns Hopkins University School of Medicine
Collaborative Group for the Study of Cryptococcal Meningitis

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	3-3/12			3-3/12
Professional	1-3/12			1-3/12
Other	2			2

Project Description:

Eleven collaborating institutions have completed the first year of a projected two-year study of cryptococcal meningitis. Goal of the study has been to determine whether a new combination 5-fluorocytosine - amphotericin B regimen would give more rapid and at least as effective therapy as amphotericin B alone, and with less permanent renal damage. Results of therapy on 24 cases have been entered on computer punch cards and analyzed with the aid of programs devised in consultation with Dr. David Alling. Early results with the combination regimen have been quite favorable, but long-term follow-up will be necessary to check for relapse and residual renal damage in both treatment groups. Transient 5-fluorocytosine induced leukopenia in three NIH study patients was studied in collaboration with Dr. Meyers (NCI) and found to be associated with accumulation of the nucleic acid precursor, dUMP, in marrow cells of all three. This is strong preliminary evidence that the drug's toxicity is not due to defective ribosomal RNA, as has been postulated, but to inhibition of thymidylate synthetase by a metabolite of 5-fluorocytosine, 5F dUMP. Drug toxicity would then be by the same mechanism as therapeutic effect, based upon preliminary studies done in collaboration with Dr. Diasio (NCI). If further study confirms these findings, it should prove possible to block the toxicity but not the chemotherapeutic effect of the drug with exogenous thymidine. As a part of analyzing 5-fluorocytosine toxicity, blood samples from study patients were bioassayed for drug content here and also assayed for 5-fluorocytosine by gas liquid chromatography at Johns Hopkins University. The GLC assay was comparable and less variable than bioassay.

Experimental, slowly lethal cryptococcosis in the strain 13 guinea pig has been found to share many features of cryptococcosis in man. Immunologic response to cryptococcal antigen fails to develop during 3-5 weeks of infection, as measured by delayed skin response, release of MIF by peripheral blood lymphocytes and lymphocyte transformation. Humoral antibody is undetectable whereas capsular polysaccharide circulates in the blood. Nonspecific anergy and lymphocytopenia is absent in these animals but is detectable in animals dying more rapidly. This model provides an opportunity to define the mechanism of anergy in cryptococcosis and measure its impact on resistance to infection. MIF analyses are being done with the aid of Dr. Balow.

A patient with severe, fatal cryptococcosis was found to have no heat labile opsonins for cryptococci, in confirmation of a report by others. Study of this patient's serum in the laboratory of Dr. Frank has shown markedly defective alternate complement pathway function, using Mg EDTA - rabbit erythrocyte lysis. Our prior demonstration of alternate pathway complement activation by cryptococcal polysaccharide and this patient's high serum polysaccharide level indicated consumption was the probable cause of the complement defect. The project will be continued.

Keyword Descriptors: Cryptococcosis, MIF, lymphocyte transformation, anergy, cryptococcin, 5-fluorocytosine, amphotericin B, complement, opsonins.

Publications:

1. Block, E.R., Bennett, J.E., Livoti, L.G., Klein, W.J., MacGregor, R.R., and Henderson, L.: The effect of hemodialysis on the plasma concentration and clearance of 5-fluorocytosine and amphotericin B in man. Ann. Intern. Med. 80: 613-617, 1974.

2. Diamond, R.D., May, J.E., Kane, M.A., Frank, M.M., and Bennett, J.E.:

The role of the classical and alternate complement pathways in host defenses against Cryptococcus neoformans infection. J. Immunol. 112: 2260-2270, 1974.

3. Young, R.D., Bennett, J.E., Geelhoed, G.W., and Levine, A.S.: Fungemia in patients with compromised host resistance. Ann. Intern. Med. 80: 605-612, 1974.

4. Bennett, J.E.: Prevention of hospital-acquired systemic mycoses. Prev. Med. 3: 515-516, 1974.

5. Bennett, J.E.: Cryptococcosis. Mucormycosis. Candidiasis. Aspergillosis. Nocardiosis. Beeson, P. and McDermott, W. (Ed.): Textbook of Medicine. Philadelphia, Pa., W. B. Saunders, 1975, pp 387-388, 449-455.

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8. Gelfand, J.A. and Bennett, J.E.: Active Histoplasma Meningitis of 22-years duration. JAMA. In press.

Project No. Z01 AI 00044-08 LCI
1. LCI
2. Bacterial Diseases Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Observations on Secretory Immunoglobulins (Includes Humans, Dogs, Rabbits, and Guinea Pigs)

Previous Serial Number: NIAID-23(c)

Principal Investigator: Dr. Herbert Y. Reynolds

Other Investigators: Dr. John A. Kazmierowski

Cooperating Units: Dr. D. L. Rosenstreich, LMI, NIDR, NIH (No Project)
Dr. N. F. Pierce, Dept. Medicine, Baltimore City Hospital, and Johns Hopkins University, Baltimore, Maryland
Dr. Myron J. Waxdal, LI, NIAID, NIH (Project No. Z01 AI 00038-03 LI)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	1-11/12	-	-	1-11/12
Professional:	11/12	-	-	11/12
Others:	1	-	-	1

Project Description:

Objectives:

1. To complete the molecular model for canine secretory IgA and identifying the assembly and interactions of secretory component (SC) and J-chain.

2. To elucidate the ultrastructure of secretory component and *in vitro* to study the susceptibility to proteolysis of dimeric serum and secretory IgA. Further, enzymes in gut epithelial cells (from *in vitro* cell cultures) responsible for synthesis of SC and for the assembly of SC and IgA are being sought.

3. To examine the gut associated lymphoid tissue (Peyer's patches) in dogs and guinea pigs for its response to bacterial antigens and for its

capacity to process antigens and to produce immunoglobulins (antibody).

4. Protective effect of secretory antibodies (IgA and IgG) in experimental gastrointestinal infections (cholera and non-bacterial infectious diarrhea).

Methods Employed:

Gel filtration and ion exchange chromatography, affinity and immunoabsorbent chromatography, standard techniques of zone electrophoresis, immunodiffusion; sedimentation velocity and equilibrium ultracentrifugation; equilibrium dialysis and fluorescence quenching for determination of binding energies; liquid scintillation spectroscopy; polyacrylamide disc gel electrophoresis; iso-electrofocusing; amino acid analysis; and amino acid sequence analysis. For intestinal lymphoid cell tissue cultures, autoradiography; immunofluorescence and stimulation of lymphocytes by mitogens including PHA, Con A, endotoxin et cetera, measuring ^{3}H-thymidine incorporation or lymphokine production (MIF).

Major Findings:

Secretory component (SC), predominantly attached to colostral and intestinal IgA through covalent (disulfide) bonding, has been dissociated, and isolated and its molecular weight of approximately 70,000 daltons established. Further isolation of J-chain, using polyacrylamide and isoelectric focusing separation techniques, from canine colostrum and serum IgM has been done. Further characterization of SC and J-chain with amino acid analysis and sequence are in progress. Likewise, intestinal IgA (Reynolds and Johnson, J. Immuno. 4: 888, 1970), is being prepared in batch quantities for use in proteolysis experiments and for use in antibody purification with Sepharose immunoadsorbents.

Peyer's patch lymphocytes have been isolated and successfully maintained in tissue culture from guinea pigs for periods exceeding seven days. These experiments faced a formidable technical problem considering the bacterial contamination present on the gut surfaces. The lymphocytes have been identified as approximately 33-55% B-type and 40% T-type. Further, the functional capacity of Peyer's patch lymphocytes have been elucidated. T-cells in Peyer's patches are capable of mounting a graft vs. host reaction to allogeneic cells and of responding to similar cell surface antigens in two way mixed lymphocyte reactions; in addition, these Peyer's patch cells can be stimulated to liberate a lymphokine which induces monocyte chemotaxis. In summary, Peyer's patch cells have been shown to have efferent and afferent functions identified with cell mediated immunity and to behave in a manner similar to other peripheral lymphoid tissue.

Antibodies to cholera toxoid have been stimulated in serum and jejunal fluids of dogs immunized parenterally or locally in Thiry-Vella gut loops. Jejunal antibodies have been found to be both IgA and IgG and to possess antitoxin activity after intrajejunal immunization. Also, it was found that passively administrated IgG antitoxin antibody, purified from immunized dogs, protected dogs from cholera infection by orogastric challenge or local challenge into Thiry-Vella jejunal loops. Thus, gastrointestinal infection or immunization results in both IgA and IgG jejunal antibody; when adequate levels of circulating humoral IgG antitoxin antibody exist in the immune host, additional resistance or protection against gastrointestinal infection occurs. It is likely that serum derived IgG antibody is important in protecting against gastrointestinal infection and works in concert with local secretory IgA antibody. Similar studies are being conducted in human volunteers infected with infectious agents which cause acute non-bacterial diarrhea.

Significance to Biomedical Research and the Program of the Institute:

These studies defining the molecular models of canine IgA's have made critical testing possible for the role of secretory piece in protecting secretory IgA from proteolysis and in a transport function. J-chain, likewise, is important in the assembly of IgA and probably IgM molecules. All these studies of canine IgA help to extend the homology of the mucosal secretory defense system in mammals and humans which is recognized as a major barrier for interaction with viral and bacterial micro-organisms by the host.

Studies on the function of gut lymphoid cells in birds and mammals, such as the Peyer's patch areas, are contradictory and may point to the tremendous species variations which will prevent easy generalities about function and significance. Therefore, each mammalian species will ultimately require study. The interaction of lymphoid cells which process antigens, such as gut bacterial antigens, and those which subsequently produce appropriate antibodies are likely to be vital in regulating the growth of normal intestinal flora and in the development of intestinal illnesses such as cholera and other diarrheal diseases. Now that these cells can be cultured in vitro and the various lymphocyte populations accurately identified, some of the confusion has been removed and hopefully more precise functions of these lymphoid aggregates can be found.

Proposed Course:

1. Further work on the structure of secretory piece and J-chain to show the interactions with IgA immunoglobulin and the elucidation of the chains' ultrastructure.

2. To elucidate the function of antigen processing cells in gut lymphoid tissue and to extend these observations to the bronchial associated lymphoid tissue.

3. To investigate the antibody response in humans following infection or immunization with cholera and non-bacterial agents causing diarrhea.

Keyword Descriptors:

1. IgA, secretory component, J-chain

2. Secretory immunoglobulins

3. Peyer's patches, identity and function of T and B lymphocytes

4. Gastrointestinal antibodies (IgG, IgA) after cholera toxoid immunization.

Publications:

1. Pierce, N.F. and Reynolds, H.Y.: Immunity to experimental cholera. I. Protective effect of humoral IgG antitoxin demonstrated by passive immunization. J. Immunol. 113: 1017-1023, 1974.

2. Levin, D.M., Rosenstreich, D.L., Wahl, S.M., and Reynolds, H.Y.: Peyer's patch lymphocytes: Demonstration of the integrity of afferent and efferent T-cell functions in the guinea pig and rat. J. Immunol. 113: 1935-1941, 1974.

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4. Thompson, R.E., Reynolds, H.Y., and Waxdal, M.J.: Structural composition of canine secretory component and immunoglobulin A. Biochemistry, 1975. In press.

Project No. Z01 AI 00045-07 LCI
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the Interaction of Antibody and Complement on the Production of Immune Damage

Previous Serial Number: NIAID-30(c)

Principal Investigator: Dr. Michael M. Frank

Other Investigators:
Dr. Jeffrey Gelfand
Dr. Anthony Fauci
Dr. Douglas Hurley
Dr. Ivan Rothman
Mr. Robert Reinhart
Miss Thelma Gaither

Cooperating Units:
Dr. E. Jaffe, NCI, NIH (no Project No.)
Dr. Ira Green, LI, NIAID, NIH (Z01 AI 00033-07 LI)
Dr. Michael Gelfand, LI, NIAID, NIH (Z01 AI 00033-07 LI)
Dr. R. Edelson, NCI, NIH (Z01 CBD 3648-03 D)
Dr. Ethan Shevach, LI, NIAID, NIH (Z01 AI 00033-07 LI)
Dr. Charles Parker, Dept. of Medicine, Washington University School of Medicine, St. Louis, Mo.
Dr. William Shearer, Dept. of Medicine, Washington University School of Medicine, St. Louis, Mo.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1-6/12	1		2-6/12
Professional	10/12	1		1-10/12
Other	8/12			8/12

Project Description:

Objectives:

The objective of this program is the long-term study and understanding of the role of complement and antibody in the production of immune injury *in vitro* and *in vivo*. To this end, a wide variety of projects have been accomplished with a number of different investigators. These projects tend to fall into three major groups. The first is cooperative study of the effect of antibody and complement on tumor cells *in vitro*. The second major study involves a host of problems related to complement receptors in tissue, their evaluation, and their function. A third set of studies involves the importance of complement in host-defense mechanisms. This year, the laboratory has been particularly interested in the effects of complement on the survival of Candida albicans *in vivo* animal models. The fourth major project concerns the study of a linkage of one of the complement structural genes, the gene for C4 synthesis on the chromosome that codes for histocompatibility type of lymphoid and some other cells.

Methods Employed:

In all of these studies, the methods employed involve purification of complement components, their use in the sequential buildup of complement intermediates on cells, the identification of sera of patients with particular complement abnormalities so that these sera can be used as reagents, and the purification of immunoglobulins of various classes by standard techniques. Methods for the titration of complement components have been developed in our laboratory as well as in other laboratories, and these are in routine use.

Major Findings:

The first project concerns studies performed in collaboration with the group at Washington in St. Louis. These studies involve the exploration of the role of antibody and complement in tumor cell growth. The studies examined the effect of specific antibody and various complement components upon the growth of mouse L cells in tissue culture. A series of striking findings were made during the course of these investigations. It had been previously discovered by the cooperating unit that antibody can increase the rate of growth of tumor cells in vitro. The effect of complement upon this phenomena was examined. When L cells were treated with anti-L cell antibody in medium containing heat inactivated fetal calf serum, nucleoside uptake and cell growth were stimulated. The response was markedly increased when fresh, unheated serum from many sources was present in the incubation medium. Using low concentrations of serum deficient in various complement components and low concentrations of antibody, no augmentation of immunostimulation was seen with serum from an individual deficient in C1R, C2, C2 and C4, or C4 alone. Moreover, no stimulation was seen in systems augmented with serum depleted in the late complement components, C3 - 9. Normal stimulation was observed in sera deficient in C5 and C6. These findings suggested that at low concentrations of antiserum, marked enhancement of tumor growth to the extent of 20 to 30 times the rate of growth without complement, appeared to occur via activation of the classical complement pathway. At high concentrations of antibody, both the classical or alternate complement pathways appeared to be involved. Stimulation was specifically restored by purified C2 in C2 deficient serum and by the addition of purified C3 to C3 - 9 deficient serum. Thus, C3 appeared to be the critical late component for the augmentation of tumor growth. Although the mechanism of stimulation is still unknown, it is likely to involve a direct effect of C3 at the level of the cell membrane.

The second series of experiments involved studies of complement receptors. These range over a wide variety of areas. For example, in collaboration with Dr. Herbert Reynolds, we have shown that the precise type of reagent used to prepare various complement intermediates is of great importance in how these intermediates react with various receptors. Thus, cells with C3b on their surface will react with many cell types which contain the C3b receptor. C3b can be cleaved by interaction with the purified C3b inactivator to C3d. Human alveolar macrophages were shown by us to have a C3d receptor. However, when the C3 and C3d were formed on the indicator cell membrane by the action of whole serum rather than the action of carefully purified subcomponents of the system, the C3d receptor was not expressed although C3d could be shown to exist on the indicator cell surfaces. This explains our in vivo findings mentioned in an accompanying report which suggests that cells coated with C3d survive normally in the circulation. In a second series of studies, our various reagents have been used to classify lymphoreticular malignancies with the group which is expert in this area in the Cancer Institute. These reagents have been shown to be of great value in classifying various lymphoreticular malignancies and even could be of value in determining prognosis in certain patient populations. Thus, it appears that patients whose lymphoid tumors express receptors for the various complement fragments and also have T cell markers, have a more serious prognosis than those patients whose cells do not express these receptors. Among the tumors studied in the past year are the childhood lymphoblastic lymphomas which are shown to have a diverse series of markers and to thereby represent a group of conditions which are closer to acute lymphocytic leukemia than had been previously realized.

Similar techniques have been used in the examination of various dermatologic conditions, and it has been possible to show that melanocytes can be identified by many of these same membrane markers and changes in the expression of membrane markers in malignant cells have also been demonstrated. Perhaps the most exciting finding in this area is the recent demonstration by Drs. Michael Gelfand, Green, and Frank that the normal human glomerulus has within it a receptor for the complement fragment C3b. The careful demonstration of this receptor was recently reported by us at the Clinical meetings, and its presence may explain the localization of complement bearing immune complexes in the kidneys of patients with a wide variety of immunologically mediated glomerulonephritis. This finding has wide applicability in the development of our further understanding of renal disease. In studies with Dr. Jeffrey Gelfand, a new and much simpler method for detecting these surface C3b markers in tissue sections was developed. This method was also presented at the Clinical meetings. In essence, fluorescenated Salmonella are incubated in fresh serum lacking easily demonstrable anti-Salmonella antibodies. Under these conditions, the alternate pathway of complement activation is initiated, and C3b is deposited upon the surface of the fluorescent bacteria. The washed C3b coated fluorescent bacteria can be used for precise immunologic localization of cells in tissue section and for the precise separation of complement bearing lymphocytes and noncomplement bearing lymphocytes in suspension. It is suspected that this technique will receive wide use.

The linkage between the genes which control complement activity and the immune response genes have also been receiving wide attention in the last year. In collaboration with Drs. Shevach and Green, we have been able to demonstrate conclusively that the gene which previous studies have shown codes for C4, the structural gene responsible for C4 synthesis, is on the same chromosome which codes for the major histocompatibility type in the guinea pig. This has wide theoretical importance in our understanding of the development of the immune response and the role of complement in host defense mechanisms in general. It is certain that this area will be explored in detail in many laboratories in the near future.

Another series of new experiments performed within the past year concern the effect of complement on the control _in vivo_ of infection with _Candida albicans_. These studies were performed in a guinea pig model in collaboration with Drs. Hurley and Fauci. It was shown that mononuclear cells can make a major contribution to the clearance of _Candida albicans_ in the absence of complement activation. In the presence of complement, the alternate pathway plays a major protective function in host defense against this organism.

Proposed Course:

The various aspects of these projects will be continued with emphasis on the pathophysiology of complement action and the role of receptors for complement fragments in the pathogenesis of disease.

Keyword Descriptors: Complement receptors, glomerulonephritis, cancer, lymphoid malignancy.

Publications:

1. Gaither, T., Alling, D., and Frank, M.M.: A new one-step method for the functional assay of the fourth component (C4) of human and guinea pig complement. _J. Immunol_. 113: 574-583, 1974.

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3. Jaffe, E.S., Shevach, E.M., Frank, M.M. and Green, I.: Leukemic reticuloendotheliosis: presence of a receptor for cytophilic antibody. _Am. J. Med_. 57: 108-114, 1974.

4. Atkinson, J.P., Shin, H., and Frank, M.M.: Metabolic behavior of C3 in normal, C4-deficient (C4d) and cortisone-treated guinea pigs. _J. Immunol_.

113: 1085-1092, 1974.

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6. Atkinson, J.P., Glew, R.H., Neva, F.A., and Frank, M.M.: Serum complement and immunity in experimental Simian malaria. II. Preferential activation of early components and failure of depletion of late components to inhibit protective immunity. J. Infect. Dis. 131: 26-33, 1975.

7. Shearer, W.T., Atkinson, J.P., Frank, M.M., and Parker, C.W.: Immunostimulation. IV. Role of complement. J. Exp. Med. 141: 736-752, 1975.

8. Frank, M.M., May, J.E., and Kane, M.A.: Studies of the biological effects of selective C4 deficiency. In Good, R. (Ed.): Proceedings of the Second International Conference on Immunodeficiency Diseases in Man. In press.

Project No. Z01 AI 00046-07 LCI
1. LCI
2. Clinical Allergy and Hypersensitivity Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Pathogenesis of Delayed Hypersensitivity

Previous Serial Number: NIAID-33(c)

Principal Investigator: Dr. Charles H. Kirkpatrick

Other investigators:
Dr. Eric Ottesen
Dr. Peter Sohnle
Dr. Stanley Chapman
Dr. Michael Frank
Mr. Terrill Smith

Cooperating Units:
Dr. Nils Axelsen, University of Copenhagen, Copenhagen, Denmark
Dr. Jeffrey Sandler, Lab. of Cell. Metab., NHLI NIH (No project no.)
Dr. Vincent Manganiello, Lab. of Cell. Metab., NHLI, NIH (No project no.)
Dr. John Gallin, Bellevue Hospital, New York, N.Y.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	3-2/12			3-2/12
Professional	2-2/12			2-2/12
Other	1			1

Project Description:

Objectives (sub-project A):

1) Characterization of cellular immune responses in patients with immune deficiency syndromes and chronic infectious diseases.

2) Investigation of the pathogenesis of acquired and congenital immune deficiency syndromes.

3) Characterization of pathways of inflammation in patients with chronic mucocutaneous candidiasis.

4) Evaluation of various methods of reconstitution of immune responses on both in vivo and in vitro cellular immune responses and the clinical course of the patients.

5) Establishment of animal models for selected infectious diseases and evaluation of immune responses during the course of the infection.

Methods Employed:

Immune responses by patients with chronic infectious diseases such as chronic mucocutaneous candidiasis have been evaluated by in vivo and in vitro techniques. Previous studies (summarized in earlier annual reports) have revealed that about 75% of these patients have abnormalities in cell-mediated immunity. Recent studies in our laboratory have focused on cells, cell products and serum factors that may suppress immune responses in vitro and may be related to the pathogenesis of this disease in older children and in adults.

Histologic studies of affected skin in patients with candidiasis have shown intense infiltration with mononuclear cells. By use of fluorescein-labeled antisera we have investigated these lesions for deposits of antigen, antibody and complement.

Dialyzable transfer factor is still under study as a therapeutic adjunct for patients with chronic candidiasis and a patient with chronic Myco. battey infection.

An animal model for acute cutaneous candidiasis has been established by applying the organisms to the skin of guinea pigs and then covering the sites with an occlusive dressing. In other experiments, the relationships of immune responses to the clinical course has been studied in rats infected with the nematode, Trichinella spiralis.

Major Findings:

In patients with candidiasis since infancy, there is often evidence that the immune defect is congenital. In patients who develop candidiasis during late childhood or adult years, there is no evidence for a congenital disorder and the underlying abnormality is unknown. Searches for suppressive activities in these patients have disclosed a serum factor in one patient; this activity disappeared when the patient was treated with amphotericin B. In a second patient the lymphocytes failed to respond to candida antigen in vitro unless they were first cultured in the absence of antigen, washed and then stimulated. It is not yet clear if a critical membrane component is being shed from the cells during the period of culture or if a suppressor cell population is lost during this interval.

The studies of the immunopathology of chronic mucocutaneous candidiasis have revealed an unexpected finding. Even though the patients have high titers of anti-candida abtibodies in their serum, these antibodies are not usually present in the lesions. Instead it appears that candida directly activates the complement system via the alternate pathway and thereby incites an inflammatory response.

Support for this notion has come from the guinea pigs with experimental candidiasis. These animals also apparently activate the alternate pathway, generate chemotactic factors and respond to the superficial infection with marked inflamation. The infected sites are then sloughed and the lesions heal. This response does not appear to involve antibodies or delayed hypersensitivity.

The rat model for Trichinella infections have revealed fluctuations in in vitro lymphocyte responses. The role of suppressor cells in this phenomenon is under study.

The clinical evaluation of transfer factor continues to indicate that the agent is useful in preventing relapses from amphotericin B-induced remissions. Thus far we have found no benefits from treatment with transfer factor alone.

Significance:

The studies have established the importance of cell-mediated immunity in resistance to infections with certain opportunistic organisms. They have also shown that long lasting remissions may be obtained by a combined therapeutic approach employing antifungal antibiotics and immunologic reconstitution.

They have also revealed the mechanism for formation of the cutaneous inflammatory lesions in patients with chronic candidiasis. Thus, the patients are predisposed to infection because of the defect in cellular immunity, but production of lesions involves direct activation of the complement system.

Animal models have been developed that should facilitate future studies of the interface between susceptibility to infection and cellular immunity.

Proposed Course:

Each of the studies described above will be continued.

Keyword Descriptors:

Immunologic reconstitution; candidiasis; Trichinella spiralis;

complement pathway; and immune deficiency syndrome.

Objectives (Sub project B):

1) Characterization of the components and method of action of dialyzable transfer factor.

Methods Employed:

Crucial questions concerning dialyzable transfer factor concern its specificity (or lack of specificity) and composition. We have addressed these issues by fractionating transfer factor on gel filtration columns and assaying fractions for the ability to transfer delayed hypersensitivity. In addition, the effects of dialyzable transfer factor on cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in purified populations of leukocytes have been measured with a sensitive radioimmunoassay.

Major Findings:

A fraction of dialyzable transfer factor that transfers delayed skin reactivity has been isolated. The fraction contains a peak with an unusually high ratio of absorbance at 260 nm to absorbance at 280 nm. It has been suggested by some workers that this absorbance ratio indicated the presence of polynucleotides. However, work in our laboratory strongly suggests that this phenomenon is due to hypoxanthine.

Transfer factor produces marked increases in leukocyte cGMP and the response occurs primarily if not entirely in monocytes.

Significance:

Isolation of a single fraction of crude transfer factor with biologic activity is a major step toward identification of the chemical nature of the active material. This should help resolve the issue of specificity.

Cyclic nucleotides are known to modulate many immunologic and inflammatory responses. In general, agents that increase cGMP also increase the intensity of the response. The finding that transfer factor increases cellular cGMP may indicate a mechanism for antigen-independent amplication of immune responses by transfer factor.

Proposed Course:

The future for this work is clear. Additional purification will be acheived by new methods. The effect of cGMP on both afferent and efferent monocyte functions will be studied.

Keywork Descriptors:

Transfer factor; cyclic GMP

Publications:

1. Graw, R.G., Lohrmann, H.P., Bull, M.I., Decker, J., Herzig, G. P., Bull, J.M., Leventhal, B.G., Yankee, R.A., Herzig, R. H., Kreuger, G.R.F., Bleyer, W.A., Buja, M.L., McGinnis, M.H., Alter, H.J., Whang-Peng, J., Gralnick, H.R., Kirkpatrick, C.H., and Henderson, E.S.: Bone marrow transplantation following combination chemotherapy immunosuppression (B.A.C.T.) in patients with acute leukemia. Transplant. Proc. 6: 349-354, 1974.

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4. Axelsen, N.H., Kirkpatrick, C.H., and Buckley, R.H.: Precipitins to Candida albicans in chronic mucocutaneous candidiasis studied by crossed immunoelectrophoresis with intermediate gel. Clin. Exp. Immunol. 17: 385-394, 1974.

5. Edelson, R.L., Kirkpatrick, C.H., Shevach, E.M., Schein, P.S., Smith, R.W., Green, I., and Lutzner, M.: Preferential cutaneous infiltration by neoplastic thymus-derived lymphocytes: morphological and functional studies. Ann. Intern. Med. 80: 685-692, 1974.

6. Kirkpatrick, C.H.: Immunosuppressives. Clin. Pharmacol. Ther. 16: 982-985, 1974.

7. Kirkpatrick, C.H., Wells, S.A., Burdick, J.F., and Smith, T.K.: Effects of fetal thymus transplantation on defective cellular immunity. In Bergsma, D.(Ed.), Immunodeficiency in Man and Animals, Sunderland, MA, Sinauer Assoc., Inc. 1975, pp. 367-369.

8. Kirkpatrick, C.H.: Restoration of cell-mediated immune responses with transfer factor. In Bergsma, D.(Ed.), Immunodeficiency in Man and Animals, Sunderland, MA, Sinauer Assoc., Inc. 1975, pp. 441-444.

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10. Kirkpatrick, C.H. and Gallin, J.I.: Suppression of cellular immune responses following transfer factor. Cell. Immunol. 15: 470-474, 1975.

11. Kirkpatrick, C.H. and Gallin, J.I.: The chemotactic activity of dialyzable transfer factor. II. Further characterization of the activity *in vivo* and *in vitro*. In Bellanti, J.A. and Dayton, D. H. (Eds.): The

Phagocytic Cell in Host Resistance. New York, Raven Press, 1975, pp. 155-167.

12. Rhodes, J.: Macrophage heterogeneity in receptor activity: The activation of macrophage Fc receptor function *in vivo* and *in vitro*. *J. Immunol*. 114: 976-981, 1975.

13. Steinberg, A.D. and Kirkpatrick, C.H.: Concepts of immunologic intervention in the treatment of asthma. In Siegal, M.S. and Weiss, E.B. (Eds.), *Bronchial Asthma, Mechanisms and Therapeutics*, Little, Brown and Co. In press.

14. Kirkpatrick, C.H., Ottesen, E.A., Smith, T.K., Wells, S.A., and Burdick, J.F.: Reconstitution of defective cellular immunity with fetal thymus and dialyzable transfer factor: long term studies in a patient with chronic mucocutaneous candidiasis. *Clin. Exp. Immunol.* In press.

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18. Ottesen, E.A.: Eosinophilia and the lung. Kirkpatrick, C.H. and Reynolds, H.Y. (Eds.). In *Immunologic and Infectious Reactions in the Lung*. New York, M. Dekker. In press.

Project No. Z01 AI 00047-06 LCI
1. LCI
2. Clinical Parasitology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Clinical Studies on Patients with Known or Suspected Parasitic Diseases

Previous Serial Number: NIAID-40(c)

Principal Investigator: Dr. Franklin A. Neva

Other Investigators: Dr. Louis W. Heck
Dr. Allen P. Kaplan
Dr. Eric A. Ottesen

Cooperating Units: Dr. David W. Alling, OSD, NIAID, NIH (No project no.)
Dr. Richard E. Raizman, LPD, NIAID, NIH (Project no. Z01 AI 00102-01 LPD)
Dr. Peter F. Weller, LPD, NIAID, NIH (No project no.)
Dr. David J. Wyler, LPD, NIAID, NIH (No project no.)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1-4/12			1-4/12
Professional	1-4/12			1-4/12
Other				

Project Description:

Principal research efforts were concentrated on filariasis, with secondary emphasis on leishmaniasis and toxoplasmosis. However, as previously, a variety of other known or suspected parasitic disease entities were referred to us, especially in the outpatient service, which is being utilized increasingly.

Continued attempts were made to demonstrate histamine release from human basophils sensitized with IgE from cases of eosinophilic lung and challenged with microfilarial antigens. This approach to define the infecting species of filaria in this disorder may not be sensitive enough, since only suggestive positive results have been obtained with sera from two of the cases.

A joint LCI-LPD expedition (Ottesen, Weller and Heck) to one of the Cook Islands of the South Pacific afforded an opportunity to evaluate immunologic responses in 70 patients with a wide spectrum of manifestations of filariasis. In addition, careful observations were made on reactions to diethylcarbamazine (DEC) treatment in the same group. Immunologic assessment of these patients included immediate and delayed hypersensitivity skin tests to filarial and other antigens as well as lymphocyte responses to filarial antigens, mitogens and environmental antigens. Many of these tests were repeated after treatment with DEC. Results of the studies carried out during the Cook Islands expedition are still largely in the process of analysis from specimens brought back to Bethesda and cannot yet be summarized. However, among other things, we anticipate that this investigation will provide some of the first observations on cell-mediated immunity in human filariasis.

In connection with laboratory studies on experimental toxoplasmosis and leishmaniasis (Project No. Z01 AI 00102-01 LPD) special tests were carried out in patients encountered with these infections. These include mainly lymphocyte reactivity to specific and nonspecific antigens in leishmaniasis cases (Wyler) and special tests for circulating toxoplasma antigen in cases of toxoplasmosis (Raizman). All of the above studies will be continued.

Keyword Descriptors:

Filariasis, leishmaniasis, toxoplasmosis, IgE, diethylcarbamazine, antigens, cell-mediated immunity.

Publications:

1. Neva, F. A., Howard, W. A., Glew, R. H., Krotoski, W. A., Gam, A. A., Collins, W. E., Atkinson, J. P., and Frank, M. M.: Relationship of serum complement levels to events of the malarial paroxysm. J. Clin. Invest. 54: 451-460, 1974.

2. Neva, F. A., Kaplan, A. P., Pacheco, G., and Gray, L.: Tropical eosinophilia. A human model of parasitic immunopathology, with observations on serum IgE levels before and after treatment. J. Allergy Clin. Immunol. In press.

3. Raizman, R. E., Neva, F. A., Eckert, R. J., Duffy, P. A., and Lipshutz, W. H.: Chagasic mega-esophagus: similarity to achalasia by manometrics, radiography, and response to pneumatic dilatation. Am. J. Dig. Dis. In press.

Project No. Z01 AI 00048-05 LCI
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Pathophysiology of Autoimmune Hemolytic Anemia

Previous Serial Number: NIAID-44(c)

Principal Investigator: Dr. Michael M. Frank

Other Investigators: Dr. Charles Jaffe
Dr. Herbert Reynolds

Cooperating Units: Dr. John Atkinson, Dept. of Medicine, Barnes Hospital, St. Louis, Mo.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1-6/12			1-6/12
Professional	10/12			10/12
Other	8/12			8/12

Project Description:

Objectives:

To define the pathophysiologic function of antibody and complement on the surface of the red cells in patients with acute hemolytic anemia and to determine the effect of these substances on red cell survival. In these studies, a patient's own erythrocyte is thought of as a neutral particle, and one has the opportunity to directly test the effect of antibodies of various classes and of the various complement component proteins on the way these cells are handled in the circulation. It is anticipated that these studies will be extended to the examination of non-neutral particles; i.e., particles which themselves are seen as foreign, such as bacteria or fungi.

Methods Employed:

In the early studies, clearance of IgM and IgG coated erythrocytes were studied in a guinea pig model, and the effects of various classes of antibody on the survival of these cells were determined. It was shown that there are a whole series of differences in the way these two types of cells are handled. It was shown that complement is absolutely required for IgM antibody to produce red cell clearance. In the absence of complement activation, IgM coated cells are not cleared from the circulation. IgG antibody mediated clearance is augmented by complement. The clearance of IgM sensitized cells is accomplished by adherence to phagocytic elements within the liver and occurs very rapidly, within five or ten minutes after the injection of the sensitized erythrocytes. IgG coated cells, on the other hand, are cleared more slowly and show a linear survival pattern. IgG coated cells, even when cleared by complement dependent mechanisms, are destroyed by phagocytic elements of the spleen. After IgM coated cells are cleared, they undergo one of two fates. Either they are ingested by Kupffer cells within the liver or they are released from the surface of these cells back into the circulation where they survive normally as Coombs' positive cells. IgG coated cells are never released. This model was then extended in the case of humans. The initial studies were performed with IgM antibody in normal volunteers, and it was found that basically all of the findings which had been defined in the guinea pig model were directly applicable to the human situation. IgM coated cells were rapidly cleared by the liver. Clearance was absolutely complement dependent, since patients with the disease hereditary angioedema and no early complement components could not clear IgM coated cells from their circulation. However, in humans, a much larger proportion of the IgM coated cells were retained after liver clearance and not released back into the circulation. This type of pattern could be seen in guinea pigs if the macrophages of the animal were in a partially activated state. It was of interest to determine whether this pattern was a function of the presence of antibody on the cells leading to continued activation of complement after the cells were placed in the patient's circulation, or whether this was a consequence of the interaction of complement with the cells of the RES system *per se*. Recently, it was possible to directly address this question with the use of cold agglutinin IgM coated erythrocytes. Cold agglutinins were prepared from patients with cold agglutinin disease by repeated absorption and elution from red cells and purified by gel filtration and sucrose density gradient ultracentrifugation. They were incubated with chromated red cells from normal volunteers and fresh serum under conditions in which complement was deposited on the volunteer's own cells. On injection of the cells back into these volunteers, they survived exactly as did cells coated with IgM isoagglutinants. Thus, the shape of the survival curve was not related to the continued activation of complement by the IgM coating the cells but was a function of the interaction of the active complement fragments on the erythrocytes with cells of the reticuloendothelial system. As with IgM isoagglutinins, the cells were cleared rapidly by the liver, and a small proportion of the cells were released back into the circulation where they survived normally. These studies have underscored the role of receptors in the clearance of cells coated with antibody and complement from the circulation. Previously, it had been thought that complement-coated erythrocytes were severely damaged, and these damaged cells were detected by the liver, removed from the circulation and phagocytized. Our studies have shown that the removal of these complement-coated erythrocytes is directly dependent upon the presence of specific complement fragments on the red cell's surface and receptors for these fragments on the phagocytic cells within the liver. The most important component for the clearance of these cells appears to be the complement fragment C3b. C3b is cleaved by an enzyme inhibitor naturally present in serum, the C3 inactivator, to fragments, C3c and C3d. C3d coated cells are not cleared from the circulation in our studies, although the presence of a C3d receptor on mononuclear cells and phagocytes has been demonstrated by other workers. Moreover, it was shown in these studies that cold agglutinin on the surface of cells induced the deposition of C3 on very specific sites on the cell surface. Thus, cells from normal volunteers coated with cold agglutinin and then, in turn, with complement could be treated with the source of the C3 inactivator to convert C3b to C3d sites. Such cells could be reexposed to cold agglutinin, and no more C3b would be deposited upon the cells because all the C3b sites would be occupied by C3d. Such cells with C3d on their surface were more resistant to hemolysis than normal erythrocytes even though the former cells were Coombs positive with an antiglobulin reagent. This explains earlier findings by other workers. Interestingly, although these cells were heavily coated with C3d, IgM isoagglutinin would place C3b on different sites on the cell surface; and cells maximally coated with C3d with cold agglutinin and then reexposed to isoagglutinin were treated just as were normal cells in terms of clearance from the circulation. These studies led naturally into further exploration of the role of the C3 receptor in various situations. In collaboration with Dr. Herbert Reynolds, we studied phatocytic and binding properties of the various receptors on human alveolar macrophages. These were taken from normal volunteers during fiberoptic bronchoscopy or from patients with cancer. There was a direct correlation between in vitro binding to alveolar monolayers and clearance from the circulation. Cells coated with C3d via exposure to whole serum had cell bound C3d but no binding and no clearance. Volunteer studies could not be done with cells coated with purified components; however, it was shown that C3d placed on the cells with purified components did lead to the binding to alveolar macrophages. Thus, this receptor exists but its function is in doubt.

Proposed Course:

1) The role of IgG antibodies on the clearance of erythrocytes will be explored.

2) The possible use of these reagents in defining the status and availability of receptor sites on cells of the reticuloendothelial system in patients with a whole variety of immunologic disorders will be evaluated.

Keyword Descriptors: Hemolytic anemia, reticuloendothelial system, blood stream clearance, complement, complement receptors.

Publications:

1. Frank, M.M., Schreiber, A.D. and Atkinson, J.P.: Studies of the

interaction of antibody, complement, and macrophages in the immune clearance of erythrocytes. In Bellanti, J.A. and Dayton, D.H. (Eds.): The Phagocytic Cell in Host Resistance. New York, Raven Press, 1975, pp. 101-116.

2. Atkinson, J.P. and Frank, M.M.: The effect of bacillus Calmette Guerin induced macrophage activation on the in vivo clearance of sensitized erythrocytes. J. Clin. Invest. 53: 1742-1749, 1974.

3. Atkinson, J.P. and Frank, M.M.: Studies on the in vivo effects of antibody interaction of IgM antibody and complement in the immune clearance and destruction of erythrocytes in man. J. Clin. Invest. 54: 339-348, 1974.

4. Atkinson, J.P. and Frank, M.M.: Complement-independent clearance of IgG-sensitized erythrocytes: inhibition by cortisone. Blood 44: 629-637, 1974.

5. Atkinson, J.P. and Frank, M.M.: Cortisone inhibition of complement independent erythrocyte clearance. Blood 44: 629-637, 1974.

6. Atkinson, J.P. and Frank, M.M.: Failure of cytotoxic agents to inhibit the clearance of sensitized erythrocytes. J. Reticuloendo. Soc. 16: 122-130, 1974

Project No. Z01 AI 00049-05 LCI
1. LCI
2. Biologic Structure Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Immunobiologic and Ultrastructural Characterization of the Mechanisms of Antigen Recognition

Previous Serial Number: NIAID-46(c).

Principal Investigator: Dr. Alan S. Rosenthal

Other Investigators:
Dr. Jerrold J. Ellner
Dr. Dirk K. Greineder
Dr. Peter E. Lipsky
Dr. Marcello A. Barcinski
Dr. Lanny J. Rosenwasser
Mr. J. Thomas Blake
Mrs. Kerstin U. Cehrs

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	5-4/12	-	1	6-4/12
Professional:	4-4/12	-	1	5-4/12
Others:	1	-	-	1

Project Description:

Objectives:

1. To develop and apply techniques for the isolation of immunocompetent thymus-derived lymphocytes.

2. To characterize in a quantitative and qualitative fashion the response of such cells to a variety of *in vitro* correlates of cellular immunity such as antigen-dependent lymphocyte proliferation and macrophage inhibitory factor production.

3. To characterize the ultrastructure and physiology of these cells as contrasted with other types of lymphocytes.

4. To study the mechanism of lymphocyte recognition of and activation by antigen and mitogen using visible antigenic markers such as horseradish peroxidase (HRPO), HRPO-protein conjugates and radiolabelled antigens.

5. Characterize the role of macrophage-lymphocyte interaction in the regulation of antigen recognition by thymus-derived lymphocytes.

6. To study the cellular and molecular basis of the genetic of immune responsiveness and the role of surface membrane determinants in cell cooperation.

Methods Employed:

1. Determination of DNA synthesis of lymphocytic populations with and without addition of antigen by 4 hour pulse of ^{3}H-TdR incorporation. Results assayed by either/or both autoradiography or liquid scintillation spectrometry.

2. Preparation and isolation of partially purified population of lymphocytes from oil-induced peritoneal exudates or regional draining nodes subsequent (2-4 weeks) to immunization of inbred strain 2 and 13 guinea pigs with chemically defined antigens such as horse spleen ferritin, horseradish peroxidase or hapten protein conjugates.

3. Column purification: Peritoneal exudate cells (PEC) or lymph node cell suspensions were passed over glass bead columns and incubated at 37°C for one hour. Cells were then eluted with 10% guinea pig sera in minimal essential Eagle's media and the recovered cells studied.

4. Preparation of macrophage-rich oil-induced peritoneal exudate cells.

5. Ultrastructural analysis of antigen localization by means of autoradiography (isotopically labelled antigen) or cytochemistry (peroxidase coupled or direct use).

6. Binding of lymphocytes to a macrophage receptor can be assessed both in the presence and absence of antigen. Monolayers of guinea pig macrophages are prepared by adherence of monocyte-macrophages from oil-induced peritoneal exudates to Lab-Tek tissue culture chamber slides. After removal of non-adherent cells, 7×10^{6} lymphocytes are added to either control or antigen pulsed monolayers for varying periods of time, after which the non-macrophage bound lymphocytes are removed by washing and the monolayer fixed with 1% gluteraldehyde for light or electron microscopic analysis.

7. The uptake and fate in culture of soluble protein antigens is being studied functionally and by correlation of the state and localization of ^{125}I-labelled DNP-GPA. Additional studies with KLH, random copolymers of poly-L-glutamic acid-L-lysine and PPD are also being carried out.

8. Assay of the mixed leukocyte reaction (MLR) are carried out by assay of ^{3}H-TdR incorporation occurring when cells from a variety of strain 2 and 13 guinea pig lymphoid tissues are mixed and cultured for 5-7 days in vitro.

Major Findings:

1. Complex lymphoid cell mixtures placed over glass bead or nylon wool columns are depleted of antibody forming cells and their precursors as measured by loss of plaque forming cells (PFC'S); immunoglobulin and antigen specific receptor bearing cells as measured autoradiographically. By contrast those cells which participate in in vitro correlates of cellular immunity such as antigen-dependent lymphocyte proliferation and elaboration of macrophage inhibitory factor (MIF) are excluded from the column.

2. There appears to be within the lymphoreticular system a segregation both functionally and anatomically of immune competence. Those cells which mediate cellular immune function appear to be sequestered within inflammatory sites such as oil-induced peritoneal exudates. These sequestered cells possess enhanced immune responsiveness producing greater inhibition of macrophage migration by a factor of 10 than that of an equivalent number of lymphocytes derived from the regional nodes. Moreover, antigen recognition as indicated by new DNA synthesis in inflammatory exudate lymphocytes is 30-200 fold greater than an equivalent number of lymph node lymphocytes. The differences cannot be explained by presence or absence of macrophages, significant blastogenic factor release or desparities in the kinetics of cell responsiveness.

3. The peritoneal exudate lymphocyte have unique surface membrane changes on ultrastructural analysis.

4. The lymphocytes within the peritoneal exudate (PELS) appear to represent thymus-derived or T-lymphocytes. At least two types exist. One possesses a cell surface membrane modification, a uropod, whose formation is metabolically dependent. By contrast B lymphocytes which bear surface immunoglobulin do not appear to form uropods spontaneously.

5. Antigen recognition and activation of immunocompetent T lymphocytes is a complex multicellular event. While the exact mechanism is not established, our data strongly indicate that an initial interaction of antigen occurs with an immunologically naive macrophage prior to T-cell macrophage interaction. Thus T lymphocytes are not themselves capable of direct activation by soluble protein antigens. Studies employing metabolic inhibitors support the thesis that a physical interaction is necessary between the immunospecific T-lymphocyte and the macrophage bearing immunorelevant antigen. Histocompatibility linked membrane determinant play a rate limiting role in macrophage-lymphocyte cooperation.

6. Macrophages have been shown to possess surface membrane receptors from lymphocytes. Characterization of the kinetics, cellular specificity and

ultrastructure of this phenomenon suggests that antigen independent binding of lymphocytes to macrophages plays an important role in antigen recognition by guinea pig T lymphocytes.

7. Addition of antigen to monolayers of macrophages in the presence of specifically immune lymphocytes results in a stabilization of otherwise reversible antigen independent binding of lymphocytes to macrophages. Such antigen dependent binding as assessed at 24 hours exhibits specificity and histocompatibility dependency and appears to represent an early morphologic correlate of antigen-mediated lymphocyte proliferation (Lipsky).

8. The uptake of soluble antigens by macrophages proceeds via a complex temperature and metabolic dependent process. At least two major pathways exist. The first involves binding of the protein to the surface membrane. Following surface binding, a metabolic dependent phase results in internalization of antigen (micropinocytosis). The fate of this non-degraded protein is not fully established. The second major pathway appears to involve macropinocytosis (fluid phase) probably representing at least 80% of the total uptake of protein. This later leads to lysosomal fusion and degradation to free amino acids and peptides. At present the micropinocytotic pathways appears to be immunologically relevant. Correlative functional and labelling studies indicates that immunogenecity of macrophage bound proteins decays with a half-life of approximately two days. While total intact protein content with time in culture remains constant, its accessibility to the lymphocytes is progressively diminished. Characterization and localization of this protein is currently under study (Ellner).

9. The nature of the stimulating and responding cells in the mixed leukocyte reaction (MLR) is not known. It is generally agreed that the likely responding cell is a T lymphocyte. Most controversial is the nature of the stimulating cell. In view of the initial and obligatory role of the macrophage in the recognition of soluble protein antigens by T lymphocytes and support of lymphocyte viability in vitro, we have examined the role of macrophages in the MLR. We have found that in the guinea pig there exists two pathways for initiation of the MLC. The major stimulating cell is the allogeneic macrophage. A bypass mechanism exists whereby responder macrophages either directly or indirectly facilitate recognition of gene products of the membranes of non-macrophages such as allogeneic T or B lymphocytes (Greineder).

Significance to Biomedical Research and the Program of the Institute:

The isolation and characterization of an enriched population of cellular immune effector cells should provide a useful system for study of the role of the cellular immune effector cells in immunologic surveillance and their contribution to the humoral limb of host defenses. Tumor immunity and transplantation immunity have been shown to rely to a great extent upon mechanisms of cellular immune function such as cytotoxicity and elaboration of other so-called "soluble mediators". A more fundamental understanding of the immunobiology of these crucial events and morphologic correlates may provide new insights into immune competence in man and information on the mechanisms of cellular regulation of immune responsiveness. Potential approaches to therapeutic manipulation of such events would be of immediate clinical relevance.

Proposed Course:

1. Characterize the mechanism of antigen recognition by T and B lymphocytes.

2. Characterize the role of the macrophage in in vivo and in vitro initiation of cell-mediated immunity in experimental animals.

3. Characterize the localization and fate of macrophage-associated antigen in in vitro antigen-mediated lymphocyte proliferation.

4. Explore the role of histocompatibility and genetic regulation in cell-cell interactions required for expression of cellular immunity.

Keyword Descriptors:

Macrophage-lymphocyte interaction, genetic control of the immune response, antigen recognition, T lymphocytes, macrophages.

Publications:

1. Rosenthal, A.S. and Rosenstreich, D.L.: The lymphocyte uropod: A specialized surface site for immunologic recognition. In Manson, L. (Ed.): Biomembranes. New York, Plenum Press, 1974, Vol. 5, pp. 1-24.

2. West, B.C., Rosenthal, A.S., Gelb, N.A., and Kimball, H.R.: Separation and characterization of human neutrophil granules. Am. J. Path. 77: 41-61, 1974.

3. Lipsky, P.E. and Rosenthal, A.S.: Macrophage-lymphocyte interaction. II. Antigen-mediated physical interactions between immune guinea pig lymph node lymphocytes and syngeneic macrophages. J. Exp. Med. 141: 138-154, 1975.

4. Rosenthal, A.S., Blake, J.T., Ellner, J.J., Greineder, D.K., and Lipsky, P.E.: The role of macorphages in T lymphocyte antigen recognition. In Rosenthal, A.S. (Ed.): Immune Recognition. New York, Academic Press, 1975, pp. 539-554.

5. Rosenthal, A.S. (Ed.): Immune Recognition. New York, Academic Press, 1975, 855 pp.

6. Ellner, J.J. and Rosenthal, A.S.: Quantitative and immunologic aspects of the handling of 2,4 dinitrophenyl guinea pig albumin by macrophages. J. Immunol. 114: 1563-1569, 1975.

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8. Rosenthal, A.S., Lipsky, P.E., and Shevach, E.M.: Morphological and functional correlates of macrophage-lymphocyte interaction. In van Furth, R. (Ed.): Mononuclear Phagocytes in Immunity, Infection and Pathology. London, Blackwell Scientific Publications, Ltd., 1975. In press.

9. Rosenthal, A.S., Lipsky, P.E., and Shevach, E.M.: Macrophage-lymphocyte interaction and antigen recognition. Fed. Proc. In press.

10. Greineder, D.K. and Rosenthal, A.S.: Macrophage activation of allogeneic lymphocyte proliferation in the guinea pig mixed leukocyte culture. J. Immunol. In press.

11. Rosenthal, A.S., Blake, J.T., Ellner, J.J., Greineder, D.K., and Lipsky, P.E.: The function of macrophages in antigen recognition by T lymphocytes. In Nelson, D.O. (Ed.): Immunobiology of the Macrophage. New York, Academic Press, 1975. In press.

12. Lipsky, P.E. and Rosenthal, A.S.: Macrophage-lymphocyte interaction: Antigen-independent binding of guinea pig lymph node lymphocytes by macrophages. J. Immunol. In press.

13. Gallin, E.K., Wiederhold, M.L., Lipsky, P.E., and Rosenthal, A.S.: Spontaneous and induced membrane hyperpolarizations in macrophages. J. Cell Physiol. In press.

Project No. Z01 AI 00050-05 LCI
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Clinical Studies of Complement Abnormalities of Man

Previous Serial Number: NIAID-47(c)

Principal Investigator: Dr. Michael M. Frank

Other Investigators: Dr. Jeffrey A. Gelfand
Dr. Charles Jaffe
Miss Thelma Gaither

Cooperating Units: Dr. John Atkinson, Dept. of Medicine, Barnes Hospital, St. Louis, Mo.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1-10/12			1-10/12
Professional	1-2/12			1-2/12
Other	8/12			8/12

Project Description:

The general objective of this program is to define the role of complement in human illness and to develop better methods of therapy for immune damage mediated by complement. This year, major emphasis was placed on our studies of patients with hereditary angioedema. We are now following a total of about 35 families of patients with this disorder.

Methods Employed:

Purified complement components are prepared and antisera to various complement components and component inhibitors are used for estimation of component levels in human sera and other body fluids. Double-blind therapeutic studies are employed to test new methods of therapy in patients. Patients on experimental drug protocols are followed to determine signs of toxicity. New methods are devised to treat some of the acute problems associated with sudden activation of the complement system. In 1972, our group reported the first double-blind study of treatment of patients with hereditary angioedema with epsilon amino caproic acid (EACA). The group of patients on this drug has grown steadily, and we are now the major group following patients on this method of therapy in an attempt to determine the success of the therapy and the level of toxicity which can be anticipated. Virtually all patients will respond to epsilon amino caproic acid. However, a group of these patients (approximately 20 to 30%) will have significant side effects. These side effects include neuromuscular weakness which disappears when the dosage of the drug is lowered. However, this does constitute a major side effect and a major problem in therapy. Therefore, other methods of therapy are being developed. During the past year, high dose Provera therapy was examined as a possible method of treatment. The rationale for this study was the observation that patients with hereditary angioedema rarely experience attacks during their third trimester of pregnancy. Moreover, although attacks are frequently precipitated by trauma, the trauma associated with birth and delivery of a child never leads to attacks of angioedema. Progesterone levels are very high during this period. It is known that methyltestosterone will in some unknown fashion suppress or prevent attacks of hereditary angioedema, and it was of interest to determine whether Provera would do the same. A double-blind, close sequential trial was conducted with three patients on Provera therapy. Although all patients reported that period on drug was associated with somewhat fewer symptoms, this drug clearly was not as effective as EACA in preventing attacks and was termed a drug failure. Thus, we know that methyltestosterone prevents attacks of HAE, and Provera (a female hormone) in high doses does not effectively prevent attacks. Recently, an impeded androgen Danazol has become available for study. The mechanism by which methylestosterone prevents attacks is unknown. It is unknown whether this effect is due to its androgenic capacity or to some other anabolic function of the drug unassociated with its masculinizing effect. Since methyltestosterone cannot be used in women for long-term therapy, an attempt is being made to define the least toxic androgen which can be used and which is therapeutically effective. A double-blind study of Danazol has been begun within the last year, and approximately three patients are now in the midst of the double-blind, closed sequential trial. It is anticipated that 5 or 6 patients will be studied in this manner. The study design is such that the effectiveness of the drug can be determined with a high degree of precision with statistically significant data being obtained in individual patients.

The disease, HAE, responds poorly to corticosteroids, epinephrine, and antihistamine. Thus, acute attacks of HAE may be life-threatening when they involve the patient's airway. These attacks are frequently precipitated by dental work, and patients with HAE frequently note that dental surgery, especially tooth extractions, are followed by life-threatening illness. Within the past year, we have developed a method to directly deal with this problem. Patients with HAE are missing the C1 esterase inhibitor or have low levels of this protein in their serum. The C1 esterase inhibitor can be supplied by transfusions of fresh-frozen plasma. There is a theoretical objection to giving fresh-frozen plasma to these patients during attacks of HAE. Not only is the inhibitor being transfused, but also the substrate for C1 (that is, C4 and C2) are included in the fresh-frozen plasma. During attacks, patients are depleted of these substrates, and the sudden infusion of C4 and C2 could, in theory, make attacks more severe. This objection does not apply to the infusion of fresh-frozen plasma when patients are free of symptoms. We have now taken a group of 6 patients who required extensive dental surgery. These patients had had 23 episodes of dental procedures in the past, 18 of which were associated with massive swelling in the region of the mouth and pharynx and 4 of which were associated with life-threatening episodes of swelling. It was not felt to be ethical to do a double-blind study in these patients, but each received 2 units of fresh-frozen plasma on the day before the dental procedure. These 6 patients had a total of 7 dental procedures. In no case was there any swelling following dental surgery, and none of the patients had any difficulty following their extraction procedures. Thus, we feel that fresh-frozen plasma transfusion at a time when the patient is free of symptoms and before dental surgery is planned is a promising method of treatment for patients with this disorder. As part of this study, the effect of the transfusions on levels of the C1 esterase inhibitor and serum C4 were followed. In terms of a biochemical response, the group proved to be fairly heterogeneous. The rise in C1 esterase inhibitor protein and serum C4 tended to occur in parallel, and the rise lasted from a minimum of 24 hrs in one patient to a maximum of 12 days in another. There was no correlation between the extent or length of elevation of C1 esterase inhibitor or C4 and the severity of the disease. The rise in C1 esterase inhibitor was in keeping with the amount that would be expected from the amount that was transfused, assuming that most of the material remained in the intravascular compartment. The function of the C1 esterase inhibitor is to inactivate C1 which in turn inhibits its ability to cleave C4. In 6 of the 7 transfusions, the rise in serum C4 was greater than that which could be accounted for by the amount of transfused serum. This was to be expected if the C1 esterase inhibitor was serving its expected function. Of great importance was the observation that patients who had very modest rises in the level of C1 esterase inhibitor in their blood were prevented from having attacks. Some of these patients have as much as 25 or 30% of the normal level of this protein before the infusion. The previous literature would suggest that the low level of available protein functions normally in patients with HAE. It seems unlikely to us that a small rise, perhaps 2mg% in the level of a normally functioning protein would prevent attacks, and we have suggested that either the protein does not function normally in many of the patients with HAE, or the fresh-frozen plasma supplies some other factor which prevents attacks. This question is under active investigation at the present time.

Significance to Biomedical Research and the Program of the Institute

Hereditary angioedema is a rare disease. The fact that in a few short years we have developed a group of 35 families with this illness (many in the Washington area) suggests that it is by no means as uncommon as previously suspected. Moreover, it provides a model for the effect of activation of early complement components and for the study of their biological significance. It is anticipated that these patients will be used for a whole series of further studies which will help to find the biological role of complement in many clinical situations.

Keyword Descriptors: Complement, hereditary angioedema.

Publications:

1. Frank, M.M. and Atkinson, J.P.: Complement in clinical medicine. Disease-a-Month pp. 1-54, Jan., 1975.

2. Leddy, J.P., Griggs, R.C., Klemperer, M.R., and Frank, M.M.: Hereditary complement (C2) deficiency with dermatomyositis. Am. J. Med. 58: 83-91, 1975.

3. Jaffe, C.J., Atkinson, J.P., Gelfand, J.A., and Frank, M.M.: Hereditary angioedema: The use of fresh-frozen plasma for prophylaxis in patients undergoing oral surgery. Allergy Clin. Immunol. In press.

4. Green, I., Jaffe, E.S., Shevach, E.M., Edelson, R.L., Frank, M.M., and Berard, C.W.: Determination of the origin of malignant reticular cells by the use of surface membrane markers. Int. Acad. of Pathol. In press.

5. Frank, M.M., Leddy, J.P., Gaither, T., Heusinkveld, R.S., Breckenridge, R.T., and Klemperer, M.R.: Hereditary C6 deficiency in man. In Good, R. (Ed.): Proceedings of the Second International Conference on Immunodeficiency Diseases in Man. In press.

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7. Frank, M.M.: Current concepts in complement research. Scope Monograph. In press.

Project No. Z01 AI 00051-04 LCI
1. LCI
2. Bacterial Diseases Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Antibody Responses in the Respiratory Tract Following Immunization with Pseudomonas aeruginosa Antigen-Interaction of Bacteria, Respiratory Opsonins, and Alveolar Macrophages.

Previous Serial Number: NIAID-48(c)

Principal Investigator: Dr. Herbert Y. Reynolds

Other Investigators: Dr. William A. Durbin
Dr. John A. Kazmierowski

Cooperating Units: Dr. Ronald G. Crystal, Pulmonary Branch, NHLI, NIH (Project No. Z01 02407-01 PB)
Dr. Jack D. Fulmer, Pulmonary Branch, NHLI, NIH (Project No. Z01 02407-01 PB)
Dr. Robert G. Graw, Jr., Pediatric Oncology Branch, NCI, NIH (Project No. Z01 CM 06820-02 PO)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	2	1	-	3
Professional:	1	1	-	2
Others:	1	-	-	1

Project Description:

Objectives:

Pseudomonas aeruginosa is a serious opportunistic bacterial pathogen for patients with impaired host immunity due to hematological malignancies or from immunosuppressive therapy for various neoplastic or other systemic diseases. Also, patients with extensive burns or structural lung diseases, particularly those who require assisted ventilation treatment, are at high risk to develop Pseudomonas infections. Pseudomonas colonization of the respiratory tract and subsequent pneumonia is particularly troublesome because the patient's response to antibiotics is often not dramatic and the microorganisms may not be eradicated. For example, pulmonary infections in children with cystic fibrosis are difficult to cure because antibiotics such as carbenicillin and gentamicin do not diffuse into bronchial secretions in sufficient concentrations to achieve inhibitory levels against the Pseudomonas organisms. Another approach to improve therapy of these pulmonary infections is that of immunological prophylaxis through the development of specific anti-Pseudomonas antibodies in serum and in the respiratory tract. These antibodies, particularly those with opsonic antibody activity, may help the lungs' cellular defense mechanism (pulmonary macrophages) by enhancing phagocytosis and killing of Pseudomonas.

The initial phase was conducted in experimental animals and has expanded now into a more general assessment of pulmonary host defenses which includes several human experimental protocols, employing a heptavalent Pseudomonas aeruginosa vaccine (Parke-Davis, Inc. BB-IND 185), and in the study of an immunosuppressed canine model of experimental pneumonia. In many ways the clinical projects are consolidated in this experimental pneumonia model (Pseudomonas aeruginosa, serotype II), done in collaboration with Dr. David C. Dale (LCI) and Dr. Robert Graw (NCI).

Methods Employed:

Rabbits are vaccinated with a variety of monovalent and polyvalent whole-cell or lipopolysaccharide Pseudomonas vaccines to observe the development of antibody in serum and bronchial secretions. Agglutinating antibodies total titers and 2-mercaptoethanol resistant (IgG) titers, are measured. Isolation and purification of antibodies from serum and bronchial secretions employs gel filtration and ion exchange chromatography, sucrose density gradient ultracentrifugation, zone electrophoresis and other standard concentration and separation techniques. Macrophages, established in short-term cell monolayer cultures with well described techniques, are challenged with ^{14}C labeled bacteria, which have been previously opsonized; subsequent phagocytosis is measured with liquid scintillation spectroscopy and bacterial killing is assayed with standard agar pore plate cultures. Bronchial secretion proteins are quantitated by immunodiffusion precipitation methods. In addition, cellular immunity is evaluated by skin testing (histology) and in vitro culture of lymphocytes. Lymphocytes are stimulated to produce lymphokines (migration inhibition factor) and the migration of alveolar macrophages is studied.

Major Findings:

The type of specific anti-Pseudomonas antibody induced in the lungs or bronchial secretions depends upon the route of vaccination used - intramuscular (IM) or intranasal. Following parenteral (IM) immunization, IgG agglutinative and opsonic antibody is present in lung secretions which diffuses into the lung from the blood; in contrast, intranasal immunization produces secretory IgA and secretory IgG antibodies in addition to a modest systemic or serum antibody response. These bronchial antibodies have been isolated and characterized. However, IgG seems to be the more important opsonic antibody in the lung on the basis of phagocytic and killing studies done with *in vitro* alveolar macrophages and ^{14}C labeled *Pseudomonas* organisms. Cell mediated immunity (CMI) develops transiently after immunization. Intranasal immunization results in immune respiratory lymphocytes which are capable of inhibiting the migration of normal alveolar macrophages for brief intervals (2-3 weeks) after primary immunization. In contrast, systemic (parenteral) immunization does not induce respiratory tract immunity.

Results of vaccination studies in three groups of susceptible patients to *Pseudomonas* infections (those with acute leukemia, cystic fibrosis, and structural lung diseases) have been completed and show that vaccination is tolerable in these patients, if small doses of steroids are combined in the syringe with the vaccine which effectively reduces systemic reactions to the lipopolysaccharide material. A good humoral antibody response can be obtained. A decrease in the number of *Pseudomonas* infections was not found in the vaccinated (n=22) *versus* control (n=20) groups of patients with acute leukemia because of the small number of available patients. Cystic fibrosis patients (n=12) responded well to the vaccine with high titers of agglutinating antibodies; immunization has not appeared to affect the infectious course or the colonization of their respiratory tracts with *Pseudomonas*.

Significance to Biomedical Research and the Program of the Institute:

Pseudomonas infections in patients with altered immunity or with structural lung disease is a major infectious disease problem which causes a high number of acquired infections in hospitalized patients. Recognizing that patient isolation is not feasible because of the ubiquity of the *Pseudomonas* organisms, which are part of the bacterial flora of many hospitalized patients, and that antibiotic therapy may be ineffective despite the availability of good anti-*Pseudomonas* antibiotics, immunization of high risk patients with *Pseudomonas* vaccines is a reasonable addition to existing therapy. A *Pseudomonas* polyvalent vaccine, developed by Parke, Davis and Co., Detroit, Michigan, is currently under investigation (BB-IND 185).

As yet little attention has been given to the induction of protective antibodies in the respiratory tract and to the subsequent control of pulmonary colonization by *Pseudomonas*. Certainly, from the experimental animal studies described, it is possible to obtain specific bronchial antibody but to do so immunization must be prolonged enough to ensure development of IgG antibody. In the presence of the antibody which is available to opsonize *Pseudomonas* organisms, pulmonary macrophages *in vitro* are efficient in ingesting and killing these organisms. The role of cell mediated immunity, particularly in the lung, in activation of alveolar macrophages or enhancement of phagocytosis and intracellular killing must be determined.

Proposed Course:

1. Patients considered to be at high risk for *Pseudomonas* pulmonary infections, will be vaccinated with the Parke, Davis and Co., polyvalent *Pseudomonas* vaccine (Pseudogen) to determine the optimal vaccination regimen

necessary to produce specific IgG bronchial antibodies. Observations concerning the protective value of these antibodies will be made. Intranasal or possible intrabronchial immunization will be done in patients with chronic lung diseases who are at increased risk for Pseudomonas pneumonia in an attempt to maximize respiratory antibodies.

2. Continuation of the experimental pneumonia model in neutropenic and immunosuppressed dogs. Studies evaluating passive immunization with IgG agglutinating antibody will be done.

3. Studies with human respiratory macrophages and opsonic antibodies, similar to the animals experiments, will be continued. Further analysis of the cellular and protein milieu of the lower lung is underway. Patients and normal volunteers are undergoing bronchial lavage via transnasal fiberoptic bronchoscopy to obtain cells and protein components from the lower respiratory tract.

Keyword Descriptors:

1. Respiratory antibodies (IgA, IgG) following immunization with Pseudomonas lipopolysaccharide antigens.

2. Opsonic antibody (IgG) enhances alveolar macrophage phagocytosis of Pseudomonas.

3. Pseudomonas vaccine (Parke-Davis BB-IND 185) has good immunogenicity in variety of high risk patients; protection against infection, however, may be minimal.

4. Pseudomonas pneumonia in leukopenic dog model
   a) granulocyte transfusions often curative
   b) carbenicillin improves survival whereas gentamicin alone is inadequate

Publications:

1. Reynolds, H.Y. and Thompson, R.E.: Pulmonary host defenses. I. Analysis of protein and lipids in bronchial secretions and antibody responses after vaccination with Pseudomonas aeruginosa. J. Immunol. 111: 358-368, 1973.

2. Reynolds, H.Y. and Thompson, R.E.: Pulmonary host defenses. II. Interaction of respiratory antibodies with Pseudomonas aeruginosa and alveolar macrophages. J. Immunol. 111: 369-380, 1973.

3. Pennington, J.E., Reynolds, H.Y., and Carbone, P.P.: Pseudomonas pneumonia: A retrospective study of thirty-six cases. Am. J. Med. 55: 155-160, 1973.

4. Reynolds, H.Y., Thompson, R.E., and Devlin, H.B.: Development of cellular and humoral immunity in the respiratory tract of rabbits to Pseudomonas

lipopolysaccharide. J. Clin. Invest. 53: 1351-1359, 1974.

5. Pennington, J.E.: Preliminary investigations of *Pseudomonas aeruginosa* vaccine in patients with leukemia and cystic fibrosis. J. Infect. Dis. 130: S159-S162, 1974.

6. Reynolds, H.Y.: Pulmonary host defenses in rabbits following immunization with *Pseudomonas* antigens: The interaction of bacteria, antibodies, macrophages and lymphocytes. J. Infect. Dis. 130: S134-S142, 1974.

7. Dale, D.C., Reynolds, H.Y., Pennington, J.E., Elin, R.J., Pitts, T.W., and Graw, R.G.: Granulocyte transfusion therapy of experimental *Pseudomonas* pneumonia. J. Clin. Invest. 54: 664-671, 1974.

8. Dale, D.C., Reynolds, H.Y., Pennington, J.E., Elin, R.J., Pitts, T.W., and Graw, R.G.: Experimental pneumonia due to *Pseudomonas* in dogs: Controlled trial of granulocyte transfusion therapy. J. Infect. Dis. 130: S143-S144, 1974.

9. Reynolds, H.Y. and Newball, H.H.: Analysis of proteins, lipids and respiratory cells obtained from human lungs by bronchial lavage. J. Lab. Clin. Med. 84: 559-573, 1974.

10. Reynolds, H.Y.: Prevention and future control of hospital associated infections commonly caused by Gram-negative bacteria. Prev. Med. 3: 507-514, 1974.

11. Pennington, J.E., Reynolds, H.Y., Wood, R.E., Robinson, R.A., and Levine, A.S.: Use of a *Pseudomonas aeruginosa* vaccine in patients with acute leukemia and cystic fibrosis. Am. J. Med. 58: 629-636, 1975.

12. Reynolds, H.Y., Levine, A.S., Wood, R.E., Zierdt, C.H., Dale, D.C., and Pennington, J.E.: *Pseudomonas aeruginosa* infections: Persisting problems and current research to find new therapies. NIH Combined Clinical Staff Conference, Ann. Intern. Med., 1975. In press.

13. Reynolds, H.Y., Atkinson, J.P., Newball, H.H., and Frank, M.M.: Receptors for immunoglobulin and complement on human alveolar macrophages. J. Immunol., 1975. In press.

14. Reynolds, H.Y., Kazmierowski, J.A., and Newball, H.H.: Specificity of opsonic antibodies to enhance phagocytosis of *Pseudomonas aeruginosa* by human alveolar macrophages obtained from cigarette smokers and nonsmokers. J. Clin. Invest., 1975. In press.

15. Reynolds, H.Y. and Newball, H.H.: Milieu of the human respiratory tract: Analysis of cells, proteins, complement and lipids obtained from the lungs by bronchial lavage. In Kirkpatrick, C.H. and Reynolds, H.Y. (Eds.): *Immunologic and Infectious Reactions in the Lung*. New York, N.Y., M. Dekker, Inc., 1975. In press.

16. Reynolds, H.Y.: The interaction of bacteria, antibodies, macrophages, and lymphocytes in the lung following immunization with Pseudomonas antigens. In Kirkpatrick, C.H. and Reynolds, H.Y. (Eds.): Immunologic and Infectious Reactions in the Lung. New York, N.Y., M. Dekker, Inc., 1975. In press.

Project No. Z01 AI 00052-03 LCI
1. LCI
2. Bacterial Diseases Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Human Responses to *Salmonella typhosa* Vi Antigen (Clinical Research #73-I-01)

Previous Serial Number: NIAID-51(c)

Principal Investigator: Dr. Herbert Y. Reynolds

Project Description:

See the July 1, 1973 through June 30, 1974 project report. No work was done on this project during the past year. It is, therefore, terminated.

Project No. Z01 AI 00053-03 LCI
1. LCI
2. Bacterial Diseases Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Antibiotic Kinetics in Respiratory Secretions

Previous Serial Number: NIAID-52(c)

Principal Investigator: Dr. Herbert Y. Reynolds

Project Description:

See the July 1, 1973 through June 30, 1974 project report. No work was done on this project during the past year. It is, therefore, terminated.

Publications:

1. Pennington, J.E. and Reynolds, H.Y.: Concentrations of gentamicin and carbenicillin in bronchial secretions. J. Infect. Dis. 128: 63-68, 1973.

2. Pennington, J.E. and Reynolds, H.Y.: Tobramycin in bronchial secretions. Antimicrob. Agents Chemother. 4: 299-301, 1973.

3. Pennington, J.E., Block, E.R., and Reynolds, H.Y.: Five fluorocytosine and amphotericin B in bronchial secretions. Antimicrob. Agents Chemother. 6: 324-326, 1974.

4. Pennington, J.E. and Reynolds, H.Y.: Pharmacokinetics of gentamicin sulfate in bronchial secretions. J. Infect. Dis. 131: 158-162, 1975.

5. Pennington, J.E., Dale, D.C., Reynolds, H.Y., and MacLowry, J.: Effect of fever on gentamicin sulfate pharmacokinetics. J. Infect. Dis., 1975. In press.

Project No. Z01 AI 00054-03 LCI
1. LCI
2. Medical Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Etiology and Pathogenesis of Viral Gastrointestinal and Respiratory Tract Infections in Man

Previous Serial Number: NIAID-53(c)

Principal Investigator: Dr. Raphael Dolin

Other Investigators:
Dr. Millicent Coker, LCI, NIAID, NIH
Dr. Richard Reichman, LCI, NIAID, NIH
Dr. Anthony S. Fauci, LCI, NIAID, NIH
Dr. Michael J. Chusid, LCI, NIAID, NIH
Mr. Holly A. Smith, LCI, NIAID, NIH

Cooperating Units:
Dr. Albert Z. Kapikian, LID, NIAID, NIH(Z01 AI 00021-06 LID)
Dr. Frederick Klipstein, University of Rochester Medical School, Rochester, N.Y.
Dr. Arnold S. Levy, Laboratory of Digestive and Hereditary Diseases, NIAMDD, NIH
Dr. Richard G. Wyatt, LID, NIAID, NIH(Z01 AI 00021-06 LID)
Dr. Brian Murphy, LID, NIAID, NIH(Z01 Al 00024015 LID)
Dr. Jack Fullmer, NHLI, NIH(Z01 02407 01 PB)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1-10/12			1-10/12
Professional	1-2/12			1-2/12
Other	8/12			8/12

Project Description:

Objectives:

1) To define the biologic, immunologic, and epidemiologic properties of the etiologic agents of viral gastroenteritis in man.

2) To develop *in vitro* culture systems and/or laboratory animal models with which to study these agents.

3) To investigate the pathogenesis of and host response to viral infections of the gastrointestinal and respiratory tracts in man. Experimentally induced disease in normal volunteers, as well as naturally occurring cases, are studied.

4) To examine the possible roles of viral agents in acute and chronic gastrointestinal and respiratory tract diseases of unknown etiology.

5) To devise control measures based on the above information.

Methods Employed:

1) Source of Viral Agents: Seven viral agents have been obtained from naturally occurring outbreaks of viral gastroenteritis. Three have been partially characterized (the Norwalk, Hawaii and MC agents). These are maintained as infectious suspensions in veal infusion broth and serve as infectious inocula and sources of antigen. Respiratory tract viruses are isolated in tissue and organ culture from naturally occurring cases. After characterization, they are grown in large quantities to serve as standard sources of the agent for laboratory and volunteer studies.

2) In Vitro Culture Systems: Standard tissue cultures or suckling mice are employed for the isolation of conventional viral agents. Since certain fastidious agents replicate only in organ culture, human fetal intestinal and tracheal organ cultures are employed for the detection of new or occult agents. The organ cultures are examined with light, fluorescence and electron microscopy, and the explants are tested for viral interference, metabolic alterations, and enzyme production.

3) Clinical Studies

a) Volunteer Studies: After rigorous safety tests of the inocula, normal volunteers are screened for appropriate serum antibody status and are infected via oral or nasopharyngeal administration of these viruses. The volunteers are followed closely for clinical changes, and samples of blood, stool, respiratory secretions, along with selected biopsies, are obtained.

b) Naturally Occurring Cases: Similar studies are performed on outpatients and hospitalized patients referred by local physicians or the infectious disease consultation service of the LCI. In collaboration with Dr. Klipstein, mucosal biopsy specimens, stool samples and sera from patients with tropical sprue in Puerto Rico were transported to the laboratory for viral studies. In collaboration with Dr. Fullmer of NHLI, lung biopsies, as well as respiratory tract secretions, from patients with chronic interstitial pulmonary disease of unknown etiology, are provided for study. Lung biopsies from acute interstitial pulmonary ilfiltrates in host defense-compromised patients hospitalized at the Clinical Center are provided through the consultation service of the LCI.

4) Host-Defense Studies: Serum and secretory antibody responses to viral infection are examined, employing neutralization, complement fixation, hemagglutination inhibition or fluorescent antibody assays. Cell mediated immunity is investigated by mitogenic and antigenic stimulation of lymphocyte cultures. Lymphocyte subpopulations are determined by sheep red blood cell and complement receptor assays (Fauci). Nonspecific defenses such as polymorphonuclear leukocyte functions (Chusid) and interferon production are also examined.

5) Immune Electron Microscopy: Virus containing specimens(stools, respiratory secretions, tissue homogenates) are reacted with antibody-containing sera, and the aggregates are examined by negative staining under the electron microscope. This powerful technique can identify previously undected viral particles. Conversely, employing standard antigen preparations, antibodies can be detected in sera and secretions.

Major Findings:

1) Serologic Response to the Hawaii Particle: Employing immune electron microscopy, serologic responses to the previously described Hawaii particle were noted in 9 volunteers with Hawaii agent-induced disease. Serologic rises were present in a spectrum of disease ranging from frank diarrhea and vomiting to mild malaise without objective signs. Volunteers who remained asymptomatic had no antibody rises and demonstrated a trend toward higher levels of pre-existing serum antibody.

2) Host Response to Gastroenteritis Agents:

a) Cell-Mediated Immunity: Significant lymphopenia was noted in 7 of 8 volunteers with Hawaii induced disease and 8 of 9 volunteers with Norwalk induced disease. Lymphopenia lasted 24 to 48 hrs and involved all lymphocyte subpopulations. In contrast to previous findings, the lymphocytes remaining in the circulation responded normally to PHA and Con A. Absence of lymphosuppressive or lymphocytotoxic (Dr. P. Terasaki) factors in the serum, as well as the absence of *in vitro* viral-lymphocyte interactions suggested that a redistribution of circulating lymphocytes might have occurred. The intestinal histopathology of viral gastroenteritis (lymphocytic ilfiltration in the lamina propria of the small intestine - Dolin) suggested that lymphocytes may be accumulating in the gut.

b) Interferon: To test the widely suggested hypothesis that interferon may account for the rapid recovery seen in viral gastroenteritis, we examined jejunal secretions, biopsies, and aspirates for the presence of interferon by a VSV plaque reduction assay in human diploid fibroblasts. No interferon was detected before or shortly after the height of illness.

c) Polymorphonuclear (PMN) Leukocyte Function: PMN's from 7 volunteers with Hawaii induced disease demonstrated decreased stimulation of C1-glucose oxidation by phagocytosis of latex particles during acute illness. Two-weeks post illness, the PMN's from ill volunteers demonstrated increased phagocytic and bactericidal activity (Chusid).

3) Infant Diarrhea (Reovirus-like) Agent: In collaboration with Drs. Kapikian and Wyatt of LID, the recently isolated infant diarrhea agent was administered to 2 normal volunteers with high levels of pre-existing serum antibody. Neither of the volunteers demonstrated overt clinical illness, but one had a greater than four-fold rise in complement-fixing serum antibody, suggesting that viral replication had taken place. Additional studies in sero-negative volunteers are planned to elucidate the pathogenesis and mode of spread of this agent.

4) Tropical Sprue: Intestinal biopsy specimens and stool samples were obtained from 6 patients with tropical Sprue through Dr. F. Klipstein. Inoculation of all specimens into tissue cultures (Rhesus monkey kidney, Hep-2, rabbit kidney, WI-38, African green monkey kidney and human embryonic kidney), intestinal organ culture, and suckling mice failed to reveal any viral agent. Employing the patients' own sera as a source of antibody, the above samples are to be examined by immune electron microscopy.

5) Influenza: Influenza virus (H_3N_2), Udorn and Georgia strains, were administered to 10 normal volunteers at the NIAID volunteer facility at the University of Maryland, in collaboration with the Laboratory of Infectious Diseases. Eight of 10 volunteers became infected by criteria of virus shedding and antibody rises with varying degrees of illness. A mild lymphopenia was noted in 6 of 8 ill volunteers involving T, B, and null cells. Lymphocytic blastogenic responses to PHA, ConA, PWM, and SKSD over a broad dose range appeared to be mildly depressed (20-30% of pre-inoculation values). Low levels of specific antigen stimulation were seen in 4-week convalescent lymphocultures. Additional studies employing H_0N_1 influenza virus are currently underway. No consistent changes in PMN function (phagocytosis, bactericidal killing) were noted during or post illness (Chusid).

6) Chronic Pulmonary Disease: Four lung biopsies from patients with chronic interstitial pulmonary fibrosis were provided by Dr. J. Fullmer of NHLI. No viral agents were detected in these specimens. Eleven additional lung biopsies in immunosuppressed patients from the Clinical Center and Bethesda Naval Medical

Center revealed two viral agents (Varicella-Zoster and CMV).

7) Isolates from Clinical Material: In the past year, 330 clinical specimens were examined for the presence of viral agents in support of the infectious disease consultation service of the LCI or at the request of physicians at the NIH, NNMC, or in the community. 74 (22%) of these were positive for a wide variety of viral agents. Selected viral serologies were also performed to support isolation findings or for diagnosis when viral isolations were negative.

Proposed Course:

Intensive studies of the agents of viral gastroenteritis will continue. Efforts to propagate the agents in vitro in organ and tissue cultures will be pursued, and emphasis will be placed in the development of rapid, sensitive, *in vitro* detection methods. The pathogenesis of both respiratory tract and gastrointestinal viral infections in man will continue to be examined in both experimentally induced and naturally occurring disease. The possible application of anti-viral compounds to the prophylaxis and/or therapy of viral gastrointestinal (as well as respiratory tract) disease will be investigated. The possible roles of viruses in chronic G.I. and pulmonary diseases will continue to be examined.

Significance to Biomedical Research and the Program of the Institute:

Viral gastrointestinal and respiratory tract infections result in common illnesses which affect all age groups of a broad segment of the population. Identification and characterization of etiologic agents, as well as studies of the pathogenesis of these diseases in man, are first steps toward the ultimate goals of prevention and therapy. Examination of the possible roles of recognized as well as unidentified viral agents in chronic pulmonary and G.I. illness may lead to an understanding of the etiology of these diseases The newly developed techniques of immune electron microscopy and organ cultures as well as volunteer experiments, should continue to provide valuable data.

Keyword Descriptors:

Viral gastroenteritis, viral respiratory tract infections

Publications:

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2. Dolin, R., Levy, A. G., Wyatt, R. G., Thornhill, T. S. and Gardner, J. Gastroenteritis induced by the Hawaii agent: Studies of intestinal histopathology and serologic response. *Am. J. Med*. In press.

3. Dolin, R. and Baron, S. Absence of detectable interferon in jejunal biopsies, aspirates, and secretions in viral gastroenteritis. Proc. Soc. Exp. Biol. Med. In press.

4. Levy, A. G., Widerlite, L., Schwartz, C. J., Dolin, R., Blacklow, N.R., Gardner, J. O., Kimberg, D. V., and Trier, J. S. Jejunal adenylate cyclase activity in human subjects during viral gastroenteritis. Gastroenterology. In press.

Project No. Z01 AI 00055-03 LCI
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of the Immune Response in Man and Experimental Animals

Previous Serial Number: NIAID-54(c)

Principle Investigator: Dr. Anthony S. Fauci

Other Investigators:
Dr. James E. Balow
Dr. Raphael Dolin
Dr. Gary W. Hunninghake
Dr. Douglas L. Hurley
Dr. Joseph E. Parrillo
Dr. Sheldon M. Wolff
Mrs. Rhoda Hubert
Mrs. Karen Pratt

Cooperating Units: NONE

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	5-7/12			5-7/12
Professional	3-7/12			3
Other	2			2

Project Description:

Objectives:

1) To delineate the precise mechanisms whereby immunosuppressive agents such as cytotoxic drugs and corticosteroids affect the immune response in man and in experimental animals, specifically looking at *in vivo* and *in vitro* function of the monocyte and subpopulations of lympho-

cytes.

2) To correlate the specific immunosuppressive effects of cytotoxic drugs and corticosteroids with the suppression of disease activity in various inflammatory and immunologically-mediated disease states in man and experimental animals, and by doing so to aim at a greater understanding and specificity in the clinical use of these agents.

3) To separate and identify purified subpopulations of lymphoid cells in man and animals in order to more accurately define their normal functional capacity as well as to study their role in such disease states as hypersensitivity vasculitis, granuloma formation, tumor and graft rejection, and the graft-versus-host reaction. In particular, to characterize the sensitivity of these subpopulations to various immunosuppressive agents.

4) To study the mechanisms whereby altered states of immunity as described above affect host mechanisms against infections. Specifically, to develop an animal model of disseminated fungemia and bacteremia in a steroid-resistent species such as the guinea pig which is similar to man in its relative resistence to the lymphopenic effects of corticosteroids, and to use this model to characterize the cell populations which are specifically suppressed in states of altered immunity associated with disseminated infections.

Methods Employed:

1) A significant proportion of the methodology is centered around the in vitro culturing of lymphoid cells to determine their varied and numerous functional capabilities. The effects of in vivo chemotherapeutic agents as well as the effects of disease-related altered states of immunity can thus be monitored and explored.

2) Administering corticosteroids to normal volunteers and patients requiring this drug and measuring the specific effects of these agents on monocytes and lymphocyte subpopulations.

3) Labeling a subject's lymphoid cells with ^{51}chromium and reinfusing the cells back into their circulation to determine the effects of disease states and of chemotherapeutic agents on the viability and circulation patterns of these cells.

4) Identifying subpopulations of lymphoid cells by their surface markers and functional capacities, and determining the change in relative proportions of such cells in disease states and during chemotherapy. Identifying specific lymphocyte subpopulations as the cell type involved in lymphocyte-mediated disease in man and in experimentally-induced disorders in animals.

5) Producing altered states of immunity in experimental animals by

renal transplantation with long term followup (greater than 2 years) in certain inflammatory diseases in which renal failure follows previous kidney damage resulting in a patient who has attained clearcut remission and is otherwise well, but requires long-term hemodialysis.

2) The precise mechanisms whereby corticosteroids exert their anti-inflammatory and immunosuppressive effects have been under intensive investigation in experimental animals, normal man, and in various immunologically mediated and inflammatory diseases in man. Several original and clinically relevant findings have emerged. Corticosteroids were formerly thought to destroy normal lymphocytes in man. This has been shown not to be true. We have clearly demonstrated, and this has subsequently been confirmed by others that corticosteroids cause a transient decrease in circulating lymphocytes and monocytes. This lymphocytopenia is selective and differential in that certain subpopulations of cells are depleted more than others. This lymphocytopenia is caused by a corticosteroid-induced redistribution of recirculating lymphoid cells from the intravascular space to other body compartments. In addition, corticosteroids cause a selective depression of certain lymphocyte functions.

Studies involving groups of patients receiving alternate-day corticosteroid therapy have demonstrated at the cellular level the scientific basis why these regimens are effective therapeutically without significantly altering host defences against infection.

3) Studies on the specific immunosuppressive effects of cytotoxic agents in patients with various immunologically-mediated diseases have enabled us to correlate the sensitivity of various lymphoid cell populations to cytotoxic agents with the therapeutic efficacy of these agents in certain disease states.

4) The experimental model of disseminated candidiasis which we have established in the guinea pig has enabled us to dissect out the overlapping as well as the distinct mechanisms whereby cytotoxic agents and corticosteroids cause an increased susceptibility to infection. These findings have laid the framework in the attempt to understand and hopefully prevent the devastating infectious disease complications so commonly seen in patients receiving these therapeutic regimens for a variety of neoplastic and non-neoplastic disease states.

5) We have developed a method of separation of purified lymphoid cells from human bone marrow. This has allowed us to thus far make several important observations with far reaching implications ranging from studies of immunodeficiency diseases to bone marrow transplantations, graft-versus-host disease and chemotherapy of a variety of disease states. We have identified and characterized the subpopulations of lymphoid cells in the human bone marrow demonstrating that these cells are made up of approximately 20% "bone-marrow derived" or "bursa-equivalent" (B) cells, 70% lymphoid cells without detectable markers, and 10%

x-irradiation, surgical removal of lymphoid organs, or administration of immunosuppressive agents in order to determine precisely the requirement of various immunological and non-specific factors involved in host defence against infection and in immunological surveillance.

6) Developing a model of experimental disseminated fungal infection in the guinea pig in order to study the specific effects of alterations in host defences by commonly employed clinical immunosuppressive therapeutic regimens on susceptibility to infection.

7) Developing models of experimental disease states in animals such as general hypersensitivity and autoallergic disease, graft and tumor rejection and organ specific hypersensitivity disease in order to study the precise mechanisms of disease activity, the cell types involved, and the sensitivity of such disease states to various chemotherapeutic regimens.

8) Performing bone marrow aspirates in normal subjects and patients with various diseases prior to, during, and following therapy. Obtaining purified populations of lymphoid cells from these bone marrow cell suspensions by various separation techniques in order to delineate the immunocompetence of bone marrow lymphoid cells in normal man, as well as the alteration of compartmentalization and function of these cells in untreated and treated disease states.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

1) A group of patients with Wegener's granulomatosis (a disease characterized by granulomatous vasculitis involving the respiratory tract and kidneys) are being studied. Untreated, the disease is rapidly fatal, and various chemotherapeutic regimens including corticosteroids have been tried previously, and have failed. We are currently following the largest single group of such patients in the world. Over the past several years we have firmly established a particular chemotherapeutic regimen (chronic low dose cytotoxic therapy, particularly cyclophosphamide) as being highly effective in producing long term remissions which can now begin to be considered cures (greater than 5 years of freedom from disease activity). These results are now being confirmed by several centers throughout the world.

Besides serving as an excellent study model of a hypersensitivity disease and the effect of cytotoxic therapy on such a disease state, our experience with this disorder has allowed us to make several important and original observations on the effect of low dose cytotoxic therapy on subpopulations of lymphoid cells in man.

Another important contribution which the study of this disease has allowed us to make is the demonstration of the feasibility of successful thymus-derived (T) cells with characteristics similar to or identical with recirculating peripheral T cells. These findings most likely account for the T cell dependent graft-versus-host reactions seen so often with bone marrow transplantation. They may also explain the probable sequestration of lymphocyte populations during chemotherapy and certain disease states. Since the bone marrow is such a predominant organ as a source of pluripotential cells, much more clinically relevant information remains to be gathered from such studies.

6) We have studied the effect of experimentally induced viral infection in man on the distribution of circulating lymphoid cells. Infection with the Norwalk agent of viral gastroenteritis results in a transient lymphocytopenia resulting probably from a redistribution of lymphocytes from the circulation to other body compartments, including possibly the gut.

Proposed Course:

This project will be continued along the lines which have been described.

Keyword Descriptors:

Immunosuppression, corticosteroid, cytotoxic agent, lymphocyte, monocyte, subpopulation, culture, mediator, cytotoxicity, granuloma, hypersensitivity, vasculitis, Wegener's granulomatosis, infection, host defence.

Publications:

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14. Balow, J.E., D.L. Hurley, and A.S. Fauci: Cyclophosphamide suppression of established cell mediated immunity: Quantitative versus qualitative changes in lymphocyte populations. J. Clin. Invest. In press.

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16. Fauci, A.S., Dale, D.C., and Balow, J.E.: Glucocorticosteroid therapy: Mechanisms of action and clinical considerations. Ann. Intern. Med. In press.

Project No. Z01 AI 00056-03 LCI
1. LCI
2. Allergic Diseases Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Biochemical Pathways of Mediator Release and Mechanism of Tissue Injury in Allergic Diseases.

Previous Serial Number: NIAID-56(c)

Principal Investigator: Dr. Allen P. Kaplan

Other Investigators:
Dr. Louis W. Heck
Dr. Richard A. Clark
Dr. Lewis D. Yecies
Dr. Dean D. Metcalfe
Mr. Henry L. Meier
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Cooperating Units:
Dr. Franklin A. Neva, LPD, NIAID, NIH (Project No. Z01 AI 00102-01 LPD)
Dr. Fletcher Taylor, Division of Experimental Pathology, University of Oklahoma - Health Sciences Section, Oklahoma City, Oklahoma
Dr. Robert Colman, Coagulation Laboratory, University of Pennsylvania Medical School, Philadelphia, Pennsylvania
Dr. Jack Pierce, Laboratory of Phisiological Chemistry, NHLI, NIH (Project No. Z01 HL 01944-18 HE)
Dr. John I. Gallin, Chief Resident in Medicine, New York University Medical Center, New York, New York.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	6-1/12	-	-	6-1/12
Professional:	4-1/12	-	-	4-1/12
Others:	2	-	-	2

Project Description:

Objectives:

1. Determination of the interrelationship of the coagulation, fibrinolytic, kinin-generating, and complement pathways in human plasma. The relevant proteins will be purified, their mechanism of activation determined and their role in the various enzymatic pathways described. Vasoactive peptides or chemotactic factors released as a consequence of activation of the coagulation and fibrinolytic pathways will be defined. The role of plasma inhibitors to control each of these pathways will be determined.

2. Development of radioimmunoassays or radial immunodiffusion assays for assessment of the bradykinin forming pathway in human plasma focusing attention upon the early steps of the intrinsic coagulation and fibrinolytic pathways with which it is intimately associated.

3. Determination of the mechanism of activation of Hageman factor by surfaces and enzymes.

4. Determination of the mechanism of interaction of the chemotactic enzymes kallikrein and plasminogen activator with the surface of the neutrophil and monocyte.

5. To identify the chemotactic factors which are generated by activation of the Hageman factor dependent pathways in human plasma or serum.

6. To improve the methodology for the separation of prekallikrein from plasminogen proactivator, two very closely associated substrates of activated Hageman factor and compare the mechanism by which human plasma plasminogen activator converts plasminogen to plasmin with known plasminogen activators such as urokinase and streptokinase.

7. Investigation of the pathogenesis of cold urticaria, cholinergic urticaria, and chronic idiopathic urticaria and angioedema, specifically focusing upon identification of the vasoactive principle which causes the defect, identifying any immunologic mechanism which may play a role, and defining the biochemical pathway which is activated in each disorder. Particular attention is given to any role played by IgE antibody, the human basophil and mast cell, and the possibility of a platelet activating factor liberated by the basophil causing secondary degranulation of platelets with its attendant serotonin release.

Methods Employed:

Purification of plasma proteins is performed utilizing affinity chromatography, ion exchange chromatography on Quarternary aminoethyl (QAE) Sephadex and Sulphopropyl (SP) Sephadex, gel filtration, and preparative elution from gels after isoelectric focusing. These methods have been applied to the isolation of unactivated Hageman factor, Hageman factor prealbumin fragments, pre-PTA (coagulation Factor XI), prekallikrein, plasminogen proactivator, plasminogen and the plasma inhibitors α_1 antitrypsin, α_2 macroglobulin, the inhibitor of the activated first component of complement ($C\bar{1}$ INH), inter α trypsin inhibitor, α_1 chymotrypsin inhibitor, and antithrombin III. Purity is evaluated by alkaline disc gel electrophoresis, SDS gel electrophoresis, and isoelectric focusing.

Eosinophil and mononuclear chemotactic activity is assessed in Boyden chambers using partially purified cells from patients with eosinophilia or partially purified monocytes obtained with Hypaque-Ficoll cushions.

Plasma histamine and serotonin levels are determined by the radioenzyme method and plasma levels of bradykinin by radioimmunoassay.

Major Findings:

1. Unactivated human Hageman factor has been successfully purified by a combination of immunologic and affinity methods. First sheep were immunized with partially purified Hageman factor obtained by chromatography of plasma on QAE Sephadex, SP Sephadex, and elution from Concanavalin A Sepharose with 0.1M methyl α D glucoside. Then Hageman factor deficient plasma was placed through the same procedure thus isolating the major contaminants in our Hageman factor preparations. A sheep was immunized with this contaminant preparation to make an antiserum to these contaminants and the antiserum then coupled to 4B Sepharose. Passage of the Hageman factor preparation over this adsorbant removed the contaminants and immunization of a sheep with the resulting effluent yielded an antiserum to human Hageman factor. By coupling this antiserum to Sepharose an immunoadsorbant was prepared which is used to selectively purify Hageman factor from crude isolates. We increase the specific activity of Hageman factor by 2500 compared to fresh plasma and can prepare in three days quantities which previously required two months. The yield is improved as well.

2. A new plasma deficiency termed Williams Trait has been discovered which has no kininogen and diminished levels of prekallikrein and plasminogen proactivator. This plasma has abnormalities of coagulation, fibrinolysis, prekallikrein activation and generation of bradykinin. All abnormalities are corrected by reconstitution with a high molecular weight form of human kininogen which is shown to be an absolute requirement for the activation of Hageman factor. Thus we now know that the surface initiates Hageman factor activation, and this high molecular weight kininogen either activates Hageman factor, or alters the function of surface-activated Hageman factor so that it can then act upon its substrates. This step precedes the kallikrein feedback upon Hageman factor (Fletcher Factor) described last year which acts as an accelerator (Weiss, Gallin, and Kaplan, J. Clin. Invest. 53: 622-633, 1974). Our findings also resolve a controversial issue in the literature regarding the number of kininogens in plasma. It is clear that 80% is of low molecular weight. However the high molecular weight forms described by some investigators is not an aggregate of the low molecular weight form; it exists quite separately and has a special function in the initiating step of activation of coagulation, fibrinolysis, and kinin formation.

3. Plasminogen proactivator, prekallikrein, and PTA (Factor XI), three substrates of Hageman factor have been further purified and distinguished.

Monospecific antisera have been prepared to prekallikrein and PTA, and they are shown to be antigenically unique; i.e., they do not share antigenic determinants. Antisera to plasminogen proactivator have been prepared; however, clear precipitin lines have not been evident. A dimeric form of plasminogen proactivator has been found, which for the first time, completely separates it from prekallikrein both functionally and antigenically. These antisera are being utilized to assist in the purification of each factor and to prepare radial immunodiffusion assays for quantitation of their levels in human plasma.

4. Plasminogen and its interaction with human plasminogen activator has been investigated. A new low molecular weight form of plasminogen has been isolated which upon gel filtration is 45,000 vs. 90,000 for the parent molecule. Upon SDS gels, the new molecule shows two bands of 50,000 and 44,000 with native plasminogen at 94,000 suggesting that it has been cleaved in half. By activating it with streptokinase and inhibiting the plasmin with P^{32} DFP we have shown that the active site is associated with the 50,000 molecular weight band. Interaction of native plasminogen with human plasminogen activator yields a plasmin of 94,000; however, after reduction and alkylation, plasminogen does not change in size but the plasmin breaks down into bands of 65,000 and 27,500. Thus activation by plasminogen activator cleaves plasminogen into heavy and light chains which are disulfide linked in a fashion similar to activation by urokinase and streptokinase.

5. Histamine has been discovered to be selectively chemotactic for human eosinophils between 30 and 125 ng/ml. Within this dose range it deactivates the cell when preincubated with it and it does not give a dose dependent increase in random motility. The chemotactic activity and deactivation phenomena are not inhibited by H_1 or H_2 antagonists; thus some other receptor is involved. Higher doses of histamine depress cell motility to histamine or other chemotactic agents. This inhibition is H_2 receptor dependent and is mediated by cyclic AMP.

6. Study of the pathogenesis of cold urticaria has demonstrated that some patients have an IgE mediated mechanism such that the defect can be passively transferred to normal volunteers and successfully challenged with an ice cube 48 hours later. Passage of sera over an anti-IgE immunoabsorbant removes this activity. Elution of the IgE from those columns to study the mechanism of its interaction with the basophil and/or mast cell are in progress. By radioenzyme assay histamine has been shown to be a major mediator of the swelling in those patients as evaluated by submersion of their hand in ice water for five minutes and determining serial histamine levels in the draining brachial vein over a 20 minute period. Thus far determinations of serotonin and bradykinin have been negative.

Exercise challenge of some patients with cholinergic urticaria also yields gradual elevation of plasma histamine which persists for a half hour. A single patient was found to have elevated plasma serotonin levels and no histamine release; however, the serotonin levels did not closely correlate with the patients symptoms. Histamine has been identified as the mediator of swelling in hereditary vibratory angioedema, and some patients with cholinergic urticaria have been found to have some swelling after a vibratory stimulus suggesting that there may be a pathogenic relationship of these disorders.

Significance to Biomedical Research and the Program of the Institute:

The interrelated enzymatic pathways consisting of coagulation, fibrinolysis, and kinin-generation have not been extensively studied in human disease. The role played by these enzymes and their vasoactive and chemotactic byproducts are being evaluated in various types of chronic urticaria and angioedema, cold urticaria, cholinergic urticaria, and urticarial erythema multiforme as one aspect of a larger program being developed in the LCI devoted to the investigation of the etiology, pathogenesis, and therapy of allergic diseases. Hopefully identification of the various mediators and enzymatic pathways activated in these disorders will lead to an understanding of their pathophysiology and hopefully to new modes of therapy.

The discovery that histamine is chemotactic for human eosinophils may help explain the mechanism by which these cells are recruited into the vicinity of allergic reactions. Thus histamine as well as the peptides designated ECF_A together mediate this function. The net effect may depend upon the absolute quantity and ratio of these mediators since both deactivate the cell and high concentrations of histamine inhibit motility hence aided in keeping stimulated cells at the reaction site.

Proposed Course:

To continue the studies outlined above and develop the ability to evaluate all of the mediator systems in patients with allergic diseases. The mechanism of activation of Hageman factor and its substrates, the inhibition of their activities by natural inhibitors, and the generation of chemotactic factors will be pursued. Assays will be further developed and applied to measure each factor functionally and as a protein in human plasma. The role of IgE, the basophil, and the tissue mast cell in human allergic diseases will be evaluated with the emphasis upon various types of urticarias and angioedemas. The role of histamine as an eosinophil chemotactic factor will be further investigated as it relates to eosinophil function in allergy. The mechanism by which histamine is released in cold urticaria and cholinergic urticaria will be pursued as a model of "physical" allergy. An asthma program will be instituted in the fall with the addition of Dr. Michael Kaliner to the Allergic Diseases Section.

Keyword Descriptors:

Bradykinin, Hageman factor, prekallikrein, plasminogen, histamine, cold urticaria, cholinergic urticaria, angioedema, kininogen, chemotaxis.

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Project No. Z01 AI 00057-02 LCI
1. LCI
2. Clinical Mycology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Basic Studies on Pathogenic Fungi

Previous Serial Number: NIAID-57(c)

Principal Investigator: Dr. K. J. Kwon-Chung

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	3			3
Professional	1			1
Other	2			2

Project Description:

Objectives of the project cover both basic and applied aspects of various pathogenic or nonpathogenic fungi, including their morphology, pathogenicity, taxonomic position, sexual life cycle and genetics.

Morphological studies were carried out by culturing the fungi on various mycological media at different temperatures. Observations were made with a light microscope. For studies on Histoplasma capsulatum, two tester strains (+,-) were crossed and ascospores were collected. The ascospore suspension containing approximately 5,000 spores were injected into mice (1ml/mouse) intravenously. Spleen, liver and lungs were collected three weeks post injection and prepared for histopathological sections. Four different methods of culture maintenance were tested for 5 strains of H. capsulatum:

1) Maintained in yeast form on blood glucose cystein agar at 37C, transplanted once a week.

2) Maintained in mycelial form on soil-extract agar at 25C,

transplanted once a month.

3) Maintained strictly in vivo in mice.

4) Maintained in vivo and in vitro mycelial form at 25C, alternately four weeks in each state.

After one year, all five strains maintained by the different methods were tested for their asexual and sexual reproduction.

Identification of new sterols from mutants of Cryptococcus neoformans and Aspergillus fennelliae resistant to polyene antibiotics was continued from last year using gas liquid chromatography, UV spectrophotometry, nuclear magnetic resonance, infrared spectrometry and mass spectrometry.

Twenty-six strains of Sporothrix Schenckii isolated from human sporotrichosis cases and six isolates of natural isolates were compared for their temperature tolerance by incubating at various temperatures between 25°C to 40°C. The type of lesions produced by clinical isolates were traced to find the correlationship between the temperature tolerance and the lesions produced by the fungus. Two strains each from different temperature types were injected into male mice and studied their multiplication in deep organs (37°C) and testes (32 - 35°C). Pool of deep organs (lung, kidney, spleen, liver) and testes were homogenized and plated on Sabourand dextrose agar and incubated at 25°C for 10 to 14 days before counting the colony forming unit. Clinical isolates and natural isolates of Sporothrix Schenckii were compared with the isolates of Ceratocystis stenoceras in their morphology in vitro and in vivo in mice.

Results of Findings:

1) Histoplasma Capsulatum

a) Spores produced through sexual cycle (ascospores) were infective for mice as the asexual spores. The tissue form derived from the ascospores were yeast-like cells identical to those produced by asexual spores. Evidence was seen that the ascospores (1.2μ) germinate directly into yeast cells without any transitional state.

b) Capability of sexual reproduction has shown to be best preserved when cultures were maintained in the yeast form at 37°C in vitro or by animal passages. The cultures maintained in mycelial form at 25°C mutated faster than those maintained by other methods.

2) Polyene Resistance and Sterol Content of Aspergillus and Cryptococcus Neoformans

a) Aspergillus fennelliae: One more new sterol was identified as $\Delta 7,22,24(28)$ - ergostatrien - 3β-ol which has not been reported in the filamentous fungi. Analysis of sterol from revertant sectors developed from the mutant colonies contained ergosterol and showed lower resistance to amphotericin B and a higher rate of sporulation than the parent mutant strains. These results confirmed our previous observations that the presence of ergosterol was directly related to the drug susceptibility and the normal rate of sporulation in *Aspergillus* species.

b) *Cryptococcus neoformans*: Unlike various filamentous fungi, the major sterol of *C. neoformans* was fungisterol ($\Delta 7$ - ergosten - 3β-ol) and contained ergosterol as minor sterol. Two more sterols isolated from mutants resistant to polyene antibiotics were identified: (1) $\Delta 8$-(9) - ergosten - 3β-ol and (2) $\Delta 5$-8(9),22 - ergostatrien - 3β-ol. The mutants producing these two sterols were highly resistant to polyenes, avirulent for mice and failed to grow at 37°C *in vitro*.

3) *Sporothrix Schenckii*

Studies on this fungus revealed that there are at least two temperature types of *S. Schenckii* (35°C, 38°C) causing human sporotrichosis. They are morphologically indistinguishable *in vivo* or *in vitro*. The 35C type cause only skin lesions without involving lymphatic spread or deep organs. The 38C type produce both cutaneous or deep lesions but still grow better in the cooler part of the body. Comparison between strains of *Sporothrix Schenckii* and *Ceratocystis Stenoceras* indicated that *S. Schenckii* strains are not the asexual mutants of *C. Stenoceras*. The information obtained through this project will be helpful in understanding the pathogenicity and the basic biological characteristics of *Histoplasma capsulatum* and *Sporothrix Schenckii*. Sterol analysis and the drug resistance in *Cryptococcus neoformans* and *Aspergillus fennelliae* will elucidate the relationship between sterol synthesis and drug resistance in fungi. Major effort will be directed to investigate the sexual cycle of *Cryptococcus neoformans* during the next fiscal year.

Keyword Descriptors: *Histoplasma capsulatum*, sexual reproduction, mutation, *Cryptococcus neoformans*, *Aspergillus fennelliae*, sterol synthesis, polyene resistance, *Sporothrix Schenckii*, *Ceratocystis stenoceras*.

Publications:

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9. Meyers, W.M., Dooley, J.R., and Kwon-Chung, K.J.: Mycotic granuloma caused by *Phialophora repens*. *Am. J. Clin. Path*. In press.

Project No. Z01 A1 00058-02 LCI
1. LCI
2. Medical Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Pathogenesis and Chemotherapy of Herpes Virus Infections in Man

Previous Serial Number: NIAID-58(c)

Principal Investigator: Dr.Raphael Dolin

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Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1-8/12			1-8/12
Professional	1			1
Other	8/12			8/12

Project Description:

Objectives

1) To determine the pathogeneis, host defense mechanisms, and natural history of herpes virus infections in man, in both host-defense compromised and intact hosts.

2) To evaluate the efficacy of antiviral agents in the treatment of

herpes virus infections in man. These infections include herpes simplex, herpes zoster, cytomegalovirus and EB virus.

3) To develop in vitro methods to study the activity, mechanisms of actions, pharmacology, and interrelationships of antiviral agents.

4) To develop laboratory animal models in which to study pathogenesis of herpes virus infections and the effect of antiviral substances.

Methods Employed:

1) Herpes viruses are isolated from patients referred to the Medical Virology Section from the Clinical Center, Bethesda Naval Medical Center, and local practitioners. The viruses are isolated and characterized in human diploid fibroblasts and in human fetal tracheal and intestinal organ cultures.

2) The effect of individual and combined antiviral drugs such as ARA-A, ARA-C, IudR, and PPA on clinical isolates is studied in tissue and organ culture assays.

3) Host defense responses to herpetic viral infections are examined in the above patients. These include: (a) levels of circulating and locally produced neutralizing and complement fixing antibodies; (b) cell-mediated immune responses, i.e., skin test sensitivity, mitogen stimulation of peripheral lymphocyte cultures, and circulating B to T cell proportions; and (c) systemic and local (e.g., vesicle) interferon production.

4) The clinical efficacy of ARA-A in the treatment of herpes zoster and herpes simplex infections in the immunosuppressed is investigated in a randomized, controlled, double-blind study in collaboration with a NIAID-supported nationwide trial of which the principal investigator is project officer.

Major Findings:

1) Clinical trials of efficacy of severe herpetic infections in the immunosuppressed.

a) Over the past year, 11 immunosuppressed patients with varicella-zoster infections were studied, as part of the nationwide NIAID supported trial. ARA-A was administered at doses of 10 mg/kg/day intravenously for 5 days without significant toxicity. Since this was a crossover designed study, only the first 5 days of treatment were analyzed. When compared to placebo recipients, patients who received ARA-A excreted virus for fewer days (mean: 3.2 ± .65 vs. 4.5 ± .45 days), manifested new lesions for fewer days (3.8 vs. 4.9 days). 3 of 6 ARA-A treated patients reported a decrease in pain on treatment, while 2 of 5 placebo-treated patients noted an increase in pain on treatment. No patient deaths related to V-Z infection were noted. Though the above differences, themselves, are not statistically significantly different, they are consistent with the results of the 22-center nationwide trial which analyzed 87 patients (including the above cases), and found the above parameters to be statistically significantly improved in patients who received ARA-A (Alford: Generalized Wilcoxon Test - two-tailed analysis - all p. values $< .02$). Long term sequelae and visceral manifestations could not be analyzed because of the crossover design of the study and the infrequency of certain complications. A new randomized, placebo-controlled trial without a crossover is planned in the coming year.

b)Two patients with brain-biopsy documented herpes simplex encephalitis were treated with ARA-A (15 mg/kg/day for 10 days). One patient expired 2 weeks post treatment, with gram negative sepsis, and at post mortem showed widespread necrosis of the cerebral cortex. The other patient remained comatose throughout treatment, but subsequently recovered the majority of her neurological function over the next 6 months. A third patient with a congenital defect in cell-mediated immunity and herpes encephalitis received no treatment and died 48 hrs.post brain biopsy.

c) Four immunosuppressed patients with progressive mucocutaneous herpes simplex or with cytomegalovirus infections were treated with ARA-A. Virus excretion was decreased during treatment, but returned to high levels in CMV infection after treatment was stopped.

2) Serum levels of ARA-A: Employing the in vitro assay for antiviral activity developed in this laboratory last year, levels of ARA-A were determined in the sera of patients receiving the drug. Values obtained ranged from .8 μg/ml to 12 μg/ml, were present only during the 12 hour infusion of the drug and appeared relatively constant for each patient. Values could be determined within 24 hrs of receipt of sample and were not affected by the presence of pre-existing antibody or interferon. Therefore, this assay should provide relevant clinical information with which to adjust the dosages of antiviral drugs and will aid in the interpretation of efficacy studies (Coker).

3) Susceptibility of Epstein-Barr Virus (EBV) to antiviral compounds: A high-producer lymphoid line of EBV(P$_3$HR-1) was exposed to ARA-A in suspension culture, and the expression of EBV viral capsid antigen was assayed by indirect immunofluorescence. ARA-A at concentrations of 5 to 50 μg/ml inhibited the expression of EBV antigen with little effect on lymphoid cell growth. Concentrations above 50 μg/ml inhibited cell growth. This is the first reported effect of ARA-A on this important human pathogen. IudR had similar effects on EBV at doses of 1-2 μg/ml. The selective effects of these two compounds on EBV were not noted when low producer lines (EB-2, EB-3, Jijoye) were employed (Coker).

4) Guinea pig model for systemic herpes simplex virus infections: Three models were established in the above species: (a) Adult sero-negative animals: systemic or local administration of high titers of HSV-1 ($10^{7.5}$ TCID$_{50}$) result in a transient viremia, negligible mortality and serum antibody rises by 2 to 4 weeks post infection. (b) Adult immunosuppressed animals: Little effect on infection is noted with Cytoxan administration until doses of 200-300 mg/Kg (intraperitoneally) for 3 days

are administered. $10^{7.5}$ $TCID_{50}$ represents a lethal $dose_{50}$ in this system with high levels of virus found in the CNS, lungs, and heart. Survivors who are chronically infected appear to harbor virus primarily in the liver. (c) New born guinea pigs: new born guinea pigs inoculated intraperitoneally with $10^{6.0}$ $TCID_{50}$ ($1LD_{50}$), manifested systemic infection with rapid death, and virus was widely disseminated in CNS, lungs, and kidneys. Administration of ARA-A at 30 mg/kg/day had little effect on this infection, but administration of disodium phosphonoacetate (PPA) demonstrated moderate protection.

Significance to Biomedical Research and the Program of the Institute:

Herpes virus infections are a significant and widespread cause of morbidity in normal adults and children, as well as in neonates and in the immunosuppressed. Herpes simplex encephalitis is the most common fatal encephalitis in the United States. Herpes genitalis appears to be increasing in incidence in the United States and has been associated with cervical cancer. There is currently no treatment of established efficacy for these infections. Studies on the pathogenesis and host response in these infections should further define their risk to man, as well as suggest control measures. The availability of compounds such as ARA-A and PPA, which possess anti-herpes activity *in vitro* and which are relatively nontoxic, offer a promising modality for the treatment of these infections. Studies with these agents can serve as models for the evaluation and use of antiviral drugs which may be subsequently developed.

Proposed Course:

Clinical trials of antiviral agents will continue and be expanded. Host response in these patients will be investigated. The clinical pharmacology of antiviral drugs will be studied employing *in vitro* bio-assays. The sensitivity of further isolates of HSV, herpes zoster, CMV and EBV will be determined, along with the mechanism of action of these drugs against these viruses. The established guinea pig animal model will be studied to examine the pathogenesis, the roles of complement activation and of immunosuppression on herpesvirus infection. Antiviral compounds which are active *in vitro* will be evaluated for *in vivo* activity in this system.

Keyword Descriptors:

Herpesvirus infections, herpes zoster, encephalitis, antiviral compounds, adenine arabinoside.

Publications:

1. Boston Interhospital Virus Study Group and the NIAID-Sponsored Cooperative Antiviral Clinical Study (R. Dolin): Failure of high dose IDU in the therapy of herpes simplex virus encephalitis. *New Eng. J. Med.* 292: 599-603, 1975.

2. Dolin, [illegible] and Smith, H.S.: Antiviral activity of adenine arabinoside and I doxuridine in human fetal intestinal and tracheal organ cultures. J. Inf. Dis. In press.

3. Dolin, R. Gill, F. A., and Nahmias, A. J. Primary Herpesvirus Hominis type 1 infection in man: Evidence for venereal spread. J. Am. Ven. Dis. Assoc. In press.

4. Bagley, D. H., Alexander, J. C., Gill, V. J., Dolin, R., and Ketcham, A. S.: Late flavobacterium species meningitis after craniofacial exenteration. Arch. Int. Med. In press.

Project No. Z01 AI 00059-02 LCI
1. LCI
2. Medical Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Biology of Human and Animal Parvoviruses

Previous Serial Number: NIAID-59(c)

Principal Investigator: Dr. Raphael Dolin

Other Investigators: Dr. Richard Reichman

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Dr. Arthur S. Levine, POB, MD, NCI (No. Project No.)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1			1
Professional	4/12			4/12
Other	8/12			8/12

Project Description:

Objectives:

1. To study the biological and immunological properties of Adenovirus-associated viruses (AAV).

2. To investigate the relationship between AAV and its helper viruses, adenoviruses and herpesviruses.

3. To study the natural history of AAV infection in man, its possible role in human disease, and particularly in adenoviral and herpes virus infections.

4. To investigate possible in vitro and in vivo inhibitors of AAV and animal parvovirus replications.

Methods Employed:

The AAV are small defective viruses that are able to replicate completely only when coinfected with helper adenovirus. Partial replication (production of fluorescent stainable antigen) occurs when cultures are infected with a variety of herpesviruses. AAV infection in man appears to be widespread since 50 to 70% of young adults possess neutralizing antibodies against AAV types 1, 2, and 3. However, infection with AAV has not been etiologically associated with human disease.

A. Assay methods. Human embryonic kidney cells, (HEK), Hep - 2 cells, or K-B cells are infected with AAV and helper virus. Since AAV does not produce cytopathic effects, the viruses are assayed primarily by immunologic techniques. AAV specific antigens are detected by complement fixation (CF) and immunofluorescence (FA) tests. The presence of antigen is employed as an end point in infectivity titrations. Kilham Rat virus (KRV) and H-1 virus are assayed by cytopathic effect in rat nephroma tissue cultures.

B. Biophysical Techniques. Purified AAV, free of detectable adenovirus, is prepared by isopycnic gradient centrifugations in cesium chloride. This preparation serves as the antigen in CF tests and in the hyperimmunization of guinea pigs to produce monospecific immune sera.

C. Use of conditionally defective and hybrid viruses. Helper viruses with genetic lesions which render them conditionally defective (e.g. temperature sensitive mutants) are grown under permissive and restrictive conditions. Ad_2-SV_{40} hybrids with portions of the adenovirus genome deleted are grown in the maximum isolation facilities in Building 10 (2 E). The enhancement of AAV by these viruses is studied by the above procedures.

Major Findings:

1. Potentiation of AAV by Ad_2-SV_{40} hybrid. Ad_2-SV_{40} hybrids (ND_3, ND_4, ND_5) were provided by Dr. A. S. Levine. These contain 5-40% insertions of the SV_{40} genome, and 4-7% deletions of the adenovirus genome. In HEK cells, these three hybrid viruses potentiated AAV_1 and AAV_2. High titers of CF antigen (10^6 $TCID_{50}$/0;2 ml) as well as FA antigen were formed. Thus, the adenovirus helper function resides on the residual portion of the adenovirus genome and is not interfered with by insertions of large portions of the SV_{40} genome.

2. Adenovirus DNA Potentiates AAV

Adenovirus 2 DNA extracted by the method of Van der Eb (J. Rose) was employed to infect HEK cells. Infectious virus was noted by cytopathic effect by day 10, with fluorescent stainable adenovirus "V" antigen detectable earlier. Ad_2 DNA infected cells potentiated AAV, with both fluorescent stainable antigen and infectious virus being produced in low quantities. Restriction enzymes are now being employed to produce fragments of Ad_2 DNA, which will be tested for helper function, in an attempt to characterize the portion of the Adenovirus genome responsible for this activity.

3. Inhibition of Kilham Rat virus (KRV) replication by antiviral compounds.

Antiviral activities of ARA-A, IudR, and PPA were tested against KRV in rat nephroma tissue cultures. PPA inhibited 100 $TCID_{50}$ of KRV at concentrations of 10-20 µg/ml. The effects of ARA-A and IudR were variable, but some inhibition was noted with concentrations greater than 50 µg/ml. Studies of *in vivo* efficacy of these compounds against KRV infection in rodents is planned. These studies may have applicability to infections with human parvovirus-like agents which cannot be cultivated *in vitro*.

Significance to Biomedical Research and the Program of the Institute:

Though AAV viruses commonly infect man, their significance to human disease remains unknown. Because of the pathogenic and oncogenic potential of adenoviruses and herpes viruses, their relationship to AAV may be of medical importance. These defective viruses also provide a unique opportunity to study the biology of viral interactions, providing a powerful tool to investigate both helper and defective virus function. In addition, the suggestion that recently discovered human pathogenic viruses (Hepatitis A, Norwalk, Hawaii and MC agents) most closely resemble the parvovirus group among animal viruses, suggests that an understanding of parvovirus biology may aid in cultivation and characterization of those hitherto undetected agents.

Proposed Course:

The potential role of AAV in human disease, acting by itself or in connection with its helper viruses, will continue to be investigated. The mechanisms of helper functions of adenoviruses and herpesviruses will also continue to be studied, with particular emphasis on the use of hybrid viruses, conditional mutants, and nucleic acid fragments from helper viruses. The effect of antiviral compounds on parvovirus replication will also be examined.

Keyword Descriptors:

Parvoviruses, adenovirus-associated viruses

Publications: NONE

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LABORATORY OF INFECTIOUS DISEASES

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SUMMARY STATEMENT - Annual Report of the

Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
July 1, 1974 through June 30, 1975

The research programs of LID continue to be concerned with the natural history and prevention of the three major uncontrolled acute infectious diseases of man--acute respiratory tract disease, viral hepatitis and acute, non-bacterial gastroenteritis. Broad advances were made in each of these areas during 1974-1975.

Hepatitis

Hepatitis Type A - Until recently, little progress was made in our understanding of hepatitis A because neither the virus nor the infections caused by it could be identified. Last year we detected a virus-like particulate antigen in the stool of patients acutely ill with hepatitis A and demonstrated its temporal and serologic relationship to hepatitis A infection. The 27 nm particle is believed to be the hepatitis A virus. The antigen was detected, using the technique of immune electron microscopy, in stool samples collected only during the acute phase of infection. Using these particles as antigen, the development of antibody could be demonstrated during convalescence from hepatitis A infection. Antigen can also be detected in the liver of marmosets infected with hepatitis A and Hilleman has used such antigen for assay of antibody by complement fixation and immune adherence. Although these tests, especially the immune adherence test, are important advances in the seroepidemiologic study of hepatitis, type A, suitable marmosets for producing hepatitis A antigen are not available. For this reason we attempted to adapt the immune adherence test for use with hepatitis A antigens derived from stools. Hepatitis A antigen was purified from acute phase stool samples (obtained from infected Naval recruits involved in the San Diego hepatitis outbreak) by a combination of ultra-centrifugal and electrophoretic procedures. The resultant purified antigen was found to be immunoreactive in the immune adherence test and free of non-specific reactivity. In preliminary comparative tests, there was good agreement between serologic results obtained by immune electron microscopy and immune adherence. (Moritsugu, Purcell, Dienstag)

In collaboration with a number of other scientists we have tested sera obtained from hepatitis patients for serologic evidence of hepatitis A virus infection. Each of 42 individuals whose illness was compatible epidemiologically with hepatitis, type A developed a serologic response to hepatitis A antigen, as measured by immune electron microscopy. Included in this group of patients were individuals from 10 separate natural or experimental occurrences of hepatitis representing virtually all of the methods of exposure by which hepatitis A virus has been known to spread in the community: water-borne, food-borne, intrafamily, intrainstitutional and shell-fish associated. In contrast, only two of 103 patients with hepatitis not epidemiologically compatible with hepatitis, type A developed a serologic response. These two patients may have been infected through the use of illicit drugs. Included in the group of patients with non-A hepatitis were 30 with confirmed hepatitis, type B and 73 with non-B hepatitis. (Dienstag, Purcell, Kapikian)

Using immune electron microscopy for the detection of anti-HA, an evaluation of age-specific patterns of anti-HA was begun. Fifty-nine percent of 83 serum samples from individuals of all ages and from various parts of the United States contained anti-HA; there was a direct relationship between age and frequency of such antibody. Thus, anti-HA was uncommon in children and young adults. These findings suggest that the epidemiology of hepatitis, type A has changed in recent decades, probably as a result of improved public health measures. (Dienstag, Purcell, Kapikian)

In collaborative studies with Drs. Boggs and Conrad, we have examined restrospectively the temporal relationship of HA Ag particle shedding to the development of disease in volunteers experimentally infected with hepatitis A virus at the Joliet Prison. HA Ag was first detected in the stool several days before transaminase values became elevated and could no longer be detected by the time peak transaminase activity was reached. Thus, this study demonstrated the need for early collection of stool specimens for the demonstration of fecal HA Ag. (Dienstag, Kapikian, Purcell)

The hepatitis A antigen was reported previously by us to have a buoyant density in cesium chloride of approximately 1.4 gm/cc and a sedimentation coefficient in sucrose consistent with its size of 27 nm. We and others have recently also detected HA Ag with a density of 1.32-1.34 gm/cc. The significance of the different densities of HA Ag observed in these studies remains to be determined, but they may represent complete and incomplete forms of the virion. Such multiple densities have also been described for parvoviruses, a family of viruses that HA Ag closely resembles. (Dienstag, Purcell)

The chimpanzee appears to be an excellent model for the study of hepatitis, type A. The temporal relationships of HA Ag shedding in the stool, development of histopathologic lesions in the liver, serum transaminase elevation and development of anti-HA were studied. HA Ag particles were shed in the stool during the late prodrome and early acute phase of infection and they were no longer detectable by the time transaminase activity reached maximum levels. Similarly, anti-HA was first detected during the early acute phase, concident with the beginning of liver damage,and the level of such antibody rose slowly over the succeeding months. The early development of anti-HA suggests that immunopathologic factors may be important in the clinical expression of hepatitis, type A. The results of these studies were remarkably similar to those obtained in the study of experimental hepatitis A virus infection in volunteers. (Dienstag, Purcell)

Hepatitis B - A radioimmunoprecipitation test for antibody to hepatitis B core antigen (HB_c Ag) was developed. Cores of Dane particles were purified from human plasma and intrinsically labeled with tritiated thymidine via the DNA polymerase associated with such cores. The radioimmunoprecipitation test was found to be 300-1000 times more sensitive than complement fixation for measuring antibody to cores (anti-HB_c). (Moritsugu, Purcell)

Based upon results of studies of hepatitis B virus in chimpanzees and rhesus monkeys and results of biophysical and immunological studies on hepatitis B antigen, an experimental hepatitis B vaccine was prepared from purified hepatitis B antigen obtained from human chronic carriers of the antigen (in a collaborative study with Dr. Gerin). Immunologic stability of the antigen following exposure to several different potential methods of inactivation was studied, using immunogenicity extinction tests in guinea pigs. Immunogenicity was retained by the antigen following all inactivation methods. Inactivation with formalin was chosen, and safety tests of inactivated and uninactivated vaccine preparations were carried out in seronegative chimpanzees in collaboration with Drs. London, Soike and Allen. None of the vaccinated chimpanzees developed evidence of hepatitis B virus infection, indicating that the purification procedures removed and/or inactivated hepatitis B virus. Twenty-four weeks after vaccination, vaccinated and control chimpanzees were challenged with live hepatitis B virus. Most control chimpanzeees developed hepatitis, type B. In contrast, three chimpanzees vaccinated with an *ayw* subtype vaccine were completely resistant to infection with *ayw* virus, and one chimpanzee vaccinated with an *adr* subtype vaccine was protected from hepatitis but did experience an inapparent HBV infection characterized by development of anti-HB$_c$ and anti-HB$_s$. Thus, hepatitis B vaccine appeared to be safe and effective against hepatitis, type B. Based upon these encouraging results, larger batches of hepatitis B vaccine, of subtypes *adw* and *ayw*, are being prepared in collaboration with Dr. Gerin for subsequent expanded safety and efficacy tests in chimpanzees, to be followed by tests in man. (Purcell)

Non-A, NonB Hepatitis - The existence of post-transfusion heptitis not serologically related to hepatitis types A or B or CMV or EBV infection has now been documented. Such non-A, non-B hepatitis was found to occur, like hepatitis, type B, approximately 10 times more frequently following transfusion of commercial blood as following transfusion of voluntarily donated blood. Epidemiologically, non-A, non-B hepatitis resembles hepatitis, type B more closely than it does hepatitis type A: transmission to contacts is rare, and a proportion of cases progress to chronic hepatitis. Approximately three-quarters of icteric and anicteric hepatitis occurring in patients following corrective cardiac surgery is non-A, non-B hepatitis at present. (Purcell, Peterson, Purcell)

Acute, Infectious, Non-Bacterial Gastroenteritis

Norwalk-like virus particles associated with other outbreaks - Previously a 27 nm virus-like particle was detected in the stools of volunteers who became ill following oral administration of the Norwalk agent, a filterable transmissible agent derived from an outbreak of acute, epidemic, non-bacterial gastroenteritis in Norwalk, Ohio. The 27 nm virus-like particle was detected by immune electron microscopy (IEM) and was found to have a buoyant density of 1.38-1.41 gm/cm^3. Based upon the serologic responses to the 27 nm particle by individuals ill during the Norwalk outbreak and by volunteers who developed experimental illness it was postulated that the 27 nm particle was the etiologic agent of the Norwalk outbreak. The etiologic significance of the 27 nm particle was further strengthened during the past year by the close temporal association of illness and presence of particles in stools during experimental infection of volunteers.

Initially, 27 nm particles were not detected in stools from volunteers infected with filterable agents from 2 other outbreaks of acute gastroenteritis which occurred in Montgomery County (MC) and Hawaii (H). However, when stools from ill volunteers were treated with genetron (to effect a partial purification) and concentrated 10X it was possible to visualize Norwalk-like particles. Both the MC and H particles resembled the Norwalk particle and both had a buoyant density of 1.40 gm/cm^3. An individual ill during the MC outbreak developed a serologic response to the particle by IEM; a similar response to the H particle was documented for a family member who was ill during the Hawaii outbreak.

The relationship of the Norwalk, MC and H virus-like particles to each other and to hepatitis A was investigated by IEM using paired sera from individuals ill with gastroenteritis or hepatitis A. The Norwalk, H and MC particles were antigenically distinct from hepatitis A which resembles these agents in size and buoyant density. The Hawaii particle appeared to be distinct from the Norwalk and MC particles. Finally, the Norwalk and MC particles appeared to be related antigenically. Thus, 2 separate antigenic groups of 27 nm virus-like particles associated with acute gastroenteritis have been defined. (Wyatt, Kalica, Kapikian)

Reovirus-like agent of infantile gastroenteritis - A 70 nm reovirus-like agent, first described in Australia last year, has emerged as the major cause of serious gastroenteritis in infants and young children throughout the world. We detected the virus by electron microscopy (EM) and by IEM using stools obtained from young patients with diarrhea admitted to the Children's Hospital of D.C. last year. Since Jan., 1974 a total of 110 patients hospitalized with acute gastroenteritis at Children's Hospital D.C. were studied by IEM for the reovirus-like agent in collaboration with Drs. Kim, Parrott and Brandt. Forty-seven percent of the infants had the agent in their stools during the acute phase of illness. A winter pattern of infection was observed with the peak incidence occurring during January and February, 1974 and December, 1974, through February, 1975. During these peak months, 72% of 53 patients shed the virus. In contrast, the agent was not detected from June, 1974 through October, 1974. Thirty-one of the 40 virus positive patients from whom paired sera were available developed a rise in serum complement fixing (CF) antibody during convalescence. CF tests were performed with 2 antigens: stool filtrate from virus-positive patients served as the homologous human antigen and Nebraska calf diarrhea virus (NCDV), which is related to the human virus, served as a related antigen. The IEM technique was highly efficient diagnostically since few individuals developed a serologic response without shedding particles which were detected by electron microscopy. (Kapikian, Wyatt, Chanock)

Recently, the study of infantile diarrhea was enlarged to include older contacts from among family members. Twelve parents developed serologic evidence of infection following admission of their infant son or daughter to the hospital for reovirus-like gastroenteritis. This represents an infection rate of 50% of those parents studied. Only one of the parents developed diarrhea. Stools were available from 6 of the infected parents, but 70 nm particles were not detected. Documentation of infection among 50% of adult contacts studied is of some interest since such individuals may serve as a source of virus for transmission to infants who usually have limited contact with other infants. (Kapikian, Wyatt, Chanock)

Although certain stool filtrates containing the reovirus-like agent can be used as CF antigen for the serologic study of infection, this approach is limited since only an occassional stool contains enough particles to fix complement in our assay system. For this reason an alternate source of antigen for the CF test was sought. The NCDV, which is related antigenically to the reovirus-like agent, was evaluated since the bovine virus grows in bovine kidney tissue culture and thus large amounts of antigen can be prepared in the laboratory. When tissue culture harvests of NCDV were concentrated 25X, antigen activity was detected using convalescent sera from calves infected with the virus. Also, infected calves developed a specific CF antibody response to NCDV antigen. The NCDV antigen also detected a serologic response when paired sera from reovirus-like agent positive infants were tested by CF. The NCDV antigen appeared to be approximately 75% as efficient as the human virus for serodiagnosis of infection with the latter virus. (Kapikian, Cline, Kalica)

Study of the reovirus-like agent in human fetal intestinal organ culture has proceeded rather slowly because of a scarcity of fetal tissue. The human reovirus-like agent was passaged successfully 4 times in the organ culture system, but the virus did not infect large areas of the intestinal epithelium. Instead, only small foci or single cells were involved. Only 3 of 15 virus particle containing filtrates proved to be infectious for intestinal organ culture. An indirect immunofluorescence technique which employed sections of infected intestinal organ culture as the source of antigen, was used to assay for antibody to the reovirus-like agent. This assay system was as efficient as CF for detection of serologic responses to the reovirus-like agent. (Wyatt)

Two serologic surveys for prevalence of antibody to the reovirus-like agent were performed. One survey was performed by indirect immunofluorescence using sections of infected fetal intestinal organ culture, while the other employed CF using the human agent and the NCDV as antigens. The results of the 2 surveys were remarkably similar: infection with the reovirus-like agent was widespread in the Washington, D.C. area and occurred commonly (60%) during the first year of life. Most children were infected by the second year of life and by the third year few children were seronegative. (Kapikian, Wyatt)

Intensive efforts are in progress to cultivate the reovirus-like agent in monolayer tissue culture. Twenty-six virus particle containing stool filtrates have been inoculated into a variety of cell cultures. Thus far, only one of the inocula has produced a transmissible effect in culture; this effect is rather subtle and consists of an increase in cell granularity. It appears to be specific and is associated with the production of 70 nm virus particles by inoculated cultures. Inoculated cultures also develop intracellular antigen which is detectable by indirect immunofluorescence.

These findings are highly encouraging but their specificity must be confirmed in order to rule out the possibility of contamination with one of related animal viruses which [illegible] also under study in the laboratory. (Wyatt, Van Kirk, Kapikian)

Efforts to induce disease in experimental animals with reovirus-like agents were highly successful during the past year. Gnotobiotic piglets developed diarrhea 30-155 hours after oral administration of a human stool filtrate containing the reovirus-like agent. This type of illness response was successfully reproduced through 5 passages in one series and 3 times in another series. Nineteen of 21 inoculated piglets developed diarrhea. Serologic responses were detected following experimental illness. Caesarian-derived-colostrum deprived calves were also infected with the human agent, but did not become ill. However, passage of stools from infected calves induced diarrheal disease in other gnotobiotic calves. These studies performed in collaboration with Dr. Mebus of the Department of Veterinary Science, University of Nebraska are highly encouraging although, to date, illness has developed only in very young calves or piglets. Attempts to induce illness at 1 month of age have failed. (Wyatt, Van Kirk)

Finally, in a collaborative study with Drs. London, Sly and Palmer rhesus monkeys were shown to be susceptible to infection. Caesarian-derived, colostrum-deprived infant monkeys developed diarrhea when given the human reovirus-like agent 7 hours after birth. The incubation period ranged from 2-5 days and 70 nm virus particles were detected in stools in association with illness. Attempts to induce illness in older monkeys have failed thus far. As in the studies with calves and piglets this is an important goal since we are anxious to be able to challenge animals at 1 to 2 months of age in order to evaluate the protective efficacy of experimental immunoprophylactic preparations administered soon after birth. (Wyatt, Van Kirk, Kapikian)

Influenza A Virus

During the past few years we have evaluated conditional lethal, temperature sensitive (ts) mutants of influenza A virus for their attenuation in man and for their suitability for use in a live virus vaccine. Mutants which are restricted in their replication in vitro at 37°C to 38°C should similarly be limited in their growth in the lower respiratory tract, the major site of significant pathology, which has a temperature of 37°C. However, such mutants should grow with reasonable efficiency in the cooler passages of the upper respiratory tract, which have a temperature of 32 to 34°C, and thereby stimulate local and systemic immunologic defense mechanisms. Furthermore, live influenza A virus vaccines could be produced rapidly by transfer of a ts defect (or ts defects) from an attenuated ts donor virus to a new antigenic variant of this virus. For this approach to succeed it is necessary that the new antigenic variant into which a ts gene (or genes) is transferred manifest a level of attenuation and antigenicity similar to its attenuated donor.

A study of the replication of the H2N2 and H3N2 ts mutants or recombinants of influenza A virus in hamster pulmonary tissue revealed that each of the viruses was restricted in growth in comparison to wild type virus. Since these mutants were either mutagenized directly or were recombinants of mutagenized virus, it was possible that genes other than the ts genes were altered by the mutagen and that these non-ts genes were responsible for the observed restriction of replication. The following observations from recent studies of ts mutants of influenza A virus in animals argues that it is the ts lesion(s) itself that specifies attenuation. (1) There was a greater restriction of replication of ts viruses in the lungs (37°C) than in the nasal turbinates (34°C). If non-ts lesions were responsible for restriction of replication, then the decrease in viral growth should be equivalent in the lungs and nasal turbinates. (2) The level of restriction of recombinant ts viruses in the lungs was a function of their shutoff temperature, i.e., the lower the shutoff temperature the lower the level of replication. (3) Wild type revertants (ts+) of the Hong Kong/68-ts-1[E] virus grew as well as the wild type virus in the hamster lung. If non-ts lesions were responsible for restriction of replication, ts+ revertants which possessed the non-ts mutations should have manifested restricted replication in comparison to the parent wild type virus. (4) Similarly, recombinant ts+ clones produced by recombination of the Hong Kong/68-ts-1[E] and Hong Kong/68-ts-2[C] viruses grew to the same level in the hamster lung as wild type virus. (5) There was a similar level of growth restriction in vivo of the Hong Kong/68-ts-1[E] and the genetically equivalent Udorn/72-ts-1[E] double lesion recombinants (clones 16 and 24) despite the fact that the latter viruses were recombinants derived from the former virus and non-ts mutations should not have been conserved during recombination. Collectively these observations suggest that it was the ts lesion(s) that specified the observed level of growth restriction. Therefore, in the future it would not be unreasonable to expect that viruses that acquire ts lesions via genetic recombination would exhibit a level of growth restriction specified by those ts lesions. Specification of attenuation by a defined genetic lesion which can be assayed for in the laboratory offers an advantage to the use of ts mutants for vaccination. Thus, ts mutants used as vaccine strains can be assessed for genetic stability during vaccine production and later during infection in man. In this manner the genetic basis for attenuation can be monitored directly during all phases of vaccine development, manufacture and usage. (Murphy, Richman, Spring, Chanock)

In previous studies it was shown that the influenza A/1968 (H3N2)-ts-1[E] recombinant exhibited the proper balance between attenuation and immunogenicity desired of a strain to be used for vaccination. Genetic analysis revealed that the Hong Kong/68-ts-1[E] virus possessed ts lesions on two segments of the influenza A virus genome and these RNA segments segregated independently of the genes that coded for the neuraminidase and the hemagglutinin glycoproteins. This property of the Hong Kong/68-ts-1[E] virus made it possible to produce recombinants possessing the ts-1[E] genetic lesions and both surface antigens of new, wild type influenza A viruses which emerged following prevalence of the Hong Kong virus. Thus, the ts lesions from the Hong Kong/68-ts-1[E] virus were transferred to the wild type influenza A/Udorn/307/72 (H3N2) virus (antigenically indistinguishable from the A/England/42/72 prototype virus). Two subsets of virus possessing the Udorn/72 hemagglutinin and the ts phenotype were characterized. The first subset, of which clones 16 and 24 were the prototypes, possessed both ts lesions (complementation groups 1 and 2) and the 38°C shutoff temperature of the Hong Kong/69-ts-1[E] parent. Evaluation in hamsters revealed that the parent Hong Kong/68-ts-1[E] virus and the genetically equivalent recombinant Udorn clones 16 and 24 were similar to each other in the following respects: (1) suppression of growth in the lungs; (2) low level of reversion to ts+ phenotype in the lungs; and (3) protection induced to homologous wild type virus challenge. The second subset, of which clone 13 was the prototype, possessed only one of the two ts lesions present in the Hong Kong/68-ts-1[E] donor and had a 39°C shutoff temperature. In hamsters, clone 13 was less stable genetically and was less restricted in replication than clones 16 and 24 which possessed both ts lesions of ts-1[E] virus.

In man, the single and double lesioned Udorn/72 recombinants exhibited a difference in virulence. The Udorn/72 clone 13 (39°C) virus retained the capacity to cause mild febrile influenzal illness, like the Hong Kong/68-ts-1[H] (39°C) and ts-2[C] (39°C) viruses, but was attenuated in comparison to wild type influenza virus. This level of attenuation could not be explained as an effect of tissue culture passage of the clone 13 virus since in an additional study, 10^4 $TCID_{50}$ of wild type Udorn/72 virus at the same passage level as the clone 13 virus produced febrile influenzal illness which was considerably more severe than the mild influenzal disease caused by 10^7 $TCID_{50}$ of the clone 13 ts recombinant. Two clones of Udorn/72-ts-1[E] virus (clone 16 and 24) which contained both ts-1[E] lesions were administered to seronegative adult volunteers and were found to be as attenuated and antigenic as the Hong Kong/68-ts-1[E] donor virus. They were satisfactorily attenuated, stable genetically, active antigenically, and they provided protection against a wild type virus challenge. These results provide further support for the concept that the ts lesions confer attenuation, and that viruses that have a specified ts lesion or set of lesions will behave predictably in man. This view gained further support from a limited trial in man of a recent ts-1[E] recombinant. The two ts lesions of ts-1[E] were transferred from the Hong Kong/68-ts-1[E] virus to a wild type influenza A/Georgia/101/1974 virus. This recombinant ts virus was suitably attenuated and antigenic when given to 4 seronegative volunteers. In contrast, the wild type Georgia/74 virus induced febrile influenzal disease. In summary, the 2 ts-1[E] lesions have been transferred 3 times to a wild type virus bearing a new hemagglutinin and in each instance the new ts recombinant exhibited a similar level of attenuation and antigenicity in seronegative adults. Thus, it seems likely that transfer of the ts-1[E] lesions to any new influenza A virus will predictably result in attenuation of the recombinant possessing the new surface antigens. (Richman, Murphy, Chanock)

Because of the need to develop effective immunoprophylaxis against influenza A virus disease in children the Hong Kong ts-1[E] recombinant was evaluated in this age group. Each of the adult vaccinees previously studied lacked serum HI antibody but did possess measurable serum anti-neuraminidase antibody (ANAB). In contrast, many young children lack both serum HI and ANAB for viruses of the H3N2 subtype. Since neuraminidase immunity is effective in reducing the clinical response to infection with virulent virus it might also do so for the attenuated ts recombinant viruses.

Indeed the full expression of residual virulence of an attenuated influenza A virus vaccine may be manifest only in serum HI and serum ANAB negative individuals, i.e., doubly seronegative persons.

In a collaborative study at Children's Hospital of D.C. (Drs. Parrott and Kim) the Hong Kong ts-1[E] recombinant was fully attenuated for serum HI antibody negative children who possessed detectable ANAB. Infection occurred but symptoms were minimal (mild rhinitis) or absent. In contrast, each of the 6 vaccinees who lacked serum antibody for both surface antigens of the virus shed a relatively larger quantity of virus over a longer interval than the first group; in addition, 4 children developed a transient febrile response during infection and 3 had mild rhinitis. Two children who lacked serum antibody to both surface antigens of influenza virus were given the influenza A/Udorn/72-ts-1[E] recombinant virus, which had the same genetic lesions as the Hong Kong/68-ts-1[E] recombinant virus. Both of the children were infected and one developed a transient fever. None of the 23 vaccinees given the Hong Kong/68-ts-1[E] or Udorn/72-ts-1[E] recombinant at Children's Hospital, D.C. developed signs or symptoms of lower respiratory tract involvement.

These findings suggest that anti-neuraminidase immunity naturally acquired from previous infection provided some protection against illness produced by the candidate vaccine virus. Only when doubly seronegative children were evaluated did it become apparent that the ts-1[E] recombinant viruses retained some virulence. It seems likely that a complete assessment of the virulence of candidate live influenza A vaccine viruses can only be made in individuals who lack immunity to both surface antigens of the influenza A virus. It is important to achieve attenuation for such persons especially if one plans to use live attenuated vaccines to control pandemic disease caused by new antigenic variants of influenza A virus. If the next pandemic shift involves the hemagglutinin and neuraminidase antigens, every member of the population will resemble the doubly seronegative child in lacking immunity to both surface antigens. In this circumstance, viruses which are acceptably attenuated for individuals with neuraminidase immunity might produce febrile responses similar to those induced by the ts-1[E] recombinants in doubly seronegative children.

The ts-1[E] recombinants were shown recently to contain discrete ts lesions which were located on two different RNA pieces of the segmented genome. Despite the existence of ts lesions on 2 separate RNA segments of the viral genome, the recombinants exhibited evidence of genetic instability during infection of 2 of 8 doubly seronegative young children during the studies at Children's Hospital of D.C. These revertants, which could not be detected in the original nasopharyngeal swab specimen, represented only a very small proportion of the virus population (10^{-2} to 10^{-3}) produced during infection of doubly seronegative children and the emergence of revertant virus under these conditions was not associated with lower respiratory tract disease. In contrast, the ts-1[E] recombinants were stable genetically when isolated from a considerably larger group of 78 adults and 6 older children who lacked serum HI antibody but possessed neuraminidase antibody. It appeared that emergence of ts+ revertants occurred only in the absence of immunity to both hemagglutinin and neuraminidase antigens; presumably only in this circumstance did vaccine virus infection occur without restriction.

In view of the occurrence of reversion and low-grade fever, studies with the ts-1[E] recombinants in children have been suspended. Although the ts-1[E] recombinants did not prove to be the ideal donor of ts lesions for a live influenza vaccine they did prove helpful in establishing new standards to be required for future ts influenza vaccine viruses —namely, genetic stability in doubly seronegative individuals and failure to induce a febrile response in such persons. Other ts mutants which contain 2 independent ts lesions in regions of the genome which do not code for the hemagglutinin or neuraminidase antigen are currently under study and several appear to be more stable genetically than the tsl[E] virus. These more stable recombinants will be evaluated in volunteers shortly. (Murphy, Richman, Spring, Chanock)

Maassab and colleagues have taken an alternate route to development of a live attenuated influenza A virus vaccine. They have developed cold adapted mutants which in serum HI antibody negative adult volunteers appear to be attenuated. A cold adapted 1960 H2N2 parental strain and a series of H3N2 cold adapted recombinants were evaluated by us recently for temperature sensitivity and genotype. The parental cold adapted mutant and 2 of the 3 recombinants were temperature sensitive as regards plaque formation and each of the ts viruses was found to have a ts lesion in complementation group I. (Spring)

Respiratory Syncytial Virus

The tsl mutant of RS virus is of special interest since this virus is almost completely attenuated for seronegative infants. Also, the mutant is moderately antigenic in such susceptible individuals. Nonetheless, ts-1 is not acceptable as a vaccine strain since it retains a small amount of residual virulence and is unstable genetically as indicated by the emergence of phenotypically altered (ts+) virus during infection of young vaccinees. Clearly, a more defective virus with greater stability is needed for vaccination against RS disease in early life. To facilitate evaluation of new candidate mutants we returned to the ts-1 mutant and undertook to define in greater detail the behavior of this virus under restrictive conditions *in vitro*. It was hoped that this information might aid us in identifying new mutants which were more defective and more attenuated than ts-1.

Growth of the ts-1 mutant occurred at the restrictive temperatures of 37°C, 38°C and 39°C; the level of replication, however, was inversely related to the incubation temperature and was significantly reduced compared either to peak wild type RS virus titer at the same temperature or the peak titer attained by the ts-1 mutant at permissive temperature. Viral growth under restrictive conditions was a function not only of the incubation temperature but also of inoculum size. Titers attained at 6 days after inoculation of replicate cell cultures with different amounts of ts-1 virus were found to be directly and linearly related to the amount of input virus; this relationship was observed at each of the restrictive temperatures tested but was not seen at permissive temperature. Suppression of viral growth observed at restrictive temperature was a consequence of two factors: (1) a decrease in production and release of infectious virus by infected cells and (2) prevention of spread of infection within the culture. Using fluorescent focus and infectious center assays it was shown that viral spread was retarded at 37°C and totally inhibited at higher incubation temperatures, as a consequence of decreased production and release of virus. However, ts-1 was able to initiate infection equally well at permissive and restrictive temperatures and the mutant persisted in infected cells under restrictive conditions in a manner which permitted expression of some viral functions without destruction of these infected cells and which allowed full expression of all viral functions, with the resultant production of infectious progeny, following shift down to permissive temperature. (Schnitzer, Richardson, Chanock)

In recent vaccine trials of the ts-1 mutant in infants and young children a small proportion of the virus recovered exhibited a partial or complete loss of temperature sensitivity, i.e., change from ts to ts+. Subsequently, the emergence of ts+ virus in cell cultures incubated at restrictive temperature was also noted. Genetic alteration in vitro, however, occurred irregularly and unpredictably. This in vitro phenomenon was investigated further after the importance of multiplicity of infection in growth of ts-1 at restrictive temperature was appreciated. As anticipated, the occurrence of genetic alteration in the ts-1 mutant was a direct function of input multiplicity. At the restrictive temperatures of 37°C and 38°C, 70-90% of cultures infected at an MOI of 0.1 pfu per cell produced ts+ virus, whereas only 10% of cultures infected at an MOI of .01 pfu per cell yielded such altered virus. Importantly, altered virus was never detected in the growth yield of virus grown at permissive temperature, although the quantity of virus produced was considered greater than that generated under restrictive conditions. Emergence of virus with altered temperature sensitivity was more frequent in cultures incubated at 37°C than in cells kept at 38°C. Genetic change in ts-1 thus appeared to be favored by those conditions which facilitated virus growth--high multiplicity of infection and the low restrictive temperature of 37°C. This suggested that genetic alteration of ts-1 was a random event which occurred with a fixed frequency during viral replication. The fact that ts+ did not develop at 32°C, although virus growth was optimal at this temperature, suggests that elevated temperature itself may play a role in increasing the frequenty of mutation.

In a continuing effort to produce a vaccine for RS virus we attempted to produce a more defective virus than ts-1 by introduction of further genetic lesions into the ts-1 virus using nitrosoguanidine (NTG) as the mutagen. The basis of selection for a more defective virus was loss of ability to produce plaques at 36°C; the ts-1 virus produces plaques at 36°C but is markedly or completely restricted in this function at 37°C. Plaques were harvested from bovine kidney (BK) cultures which had been inoculated 6 days earlier with ts-1 virus which had been treated with 15 ug/ml of NTG. A total of 427 plaque populations were tested but only 2 (#230 and 266) exhibited an alteration in plaquing efficiency at 36°C; both #230 and 266 were at least 100 fold less efficient in plaque production at 36°C than at 32°C (a permissive temperature). The 2 clones were purified by 2 additional plaque passages in bovine kidney cells and a total of 18 subclones which exhibited a 100 fold or greater reduction in plaque formation at 36°C were than characterized in greater detail. In an attempt to differentiate among these 18 subclones we tested their ability to replicate at the restrictive temperatures of 36°C, 37°C ad 38°C. When HEp-2 cells were inoculated at a multiplicity of infection (MOI) of 0.1 plaque forming unit/cell or greater, growth of each of the subclones was 10 to 100 fold lower than ts-1 at each of the restrictive temperatures. In addition, genetically altered virus able to form plaques at 37°C was detected occasionally in the growth yield under these conditions, whereas ts-1 regularly produced altered virus which induced plaques at both 37°C and 38°C. The results of these tests allowed us to select the subclones which were most defective in growth at restrictive temperatures and which were most stable genetically.

The nature of the additional ts lesions induced in the ts-1 mutant by treatment with NTG was investigated by the monolayer culture complementation test described previously. Two subclones, 230-13-5 and 266-6-5 from NTG treated ts-1 did not complement ts-1, as expected, but they did complement ts-2 and ts-7 which belong to the other 2 complementation groups of RS ts mutants. Thus, the new lesions induced in ts-1 by NTG were not in complementation groups 2 and 3.

230-13-5 and 266-6-5 were inoculated into hamsters to determine their ability to grow, to elicit an antibody response, and to protect against challenge with the wild type RS virus. Limited growth of the 230-13-5 subclone was detected in the nasal turbinates, but virus was not recovered from the lungs. Growth of 230-12-15 in the turbinates was approximately 10 fold lower than that seen with ts-1. However, this virus did induce significant resistance to subsequent challenge with wild type virus. Evidence of viral growth in the hamster was not detected with the second subclone (266-6-5). (Schnitzer, Richardson, Chanock)

M. pneumoniae

In order to evaluate the long term effects of M. pneumoniae, hamsters were infected with the wild type (ts+) and the ts-640, ts-H9 and ts-H10 mutants of the organism and studied at intervals over a 519 day period. ts-640, ts-H9 and ts-H10 mutants grew well in the lungs but did not induce any pathological changes, whereas ts+ M. pneumoniae produced significant pulmonary disease. The ts mutants proved to be stable genetically in vivo over an 18 month period of infection. Infection with the ts+, ts-640, ts-H9, or ts-H10 organisms induced significant resistance to subsequent challenge with virulent ts+ M. pneumoniae; resistance was demonstrable throughout the life span of the hamster. Animals infected with ts+ M. pneumoniae carried organisms in the upper and lower respiratory tract for a prolonged period following initial infection. Similarly, M. pneumoniae ts mutants were detected in nasal turbinates and lungs of infected hamsters for at least 465 days. Such chronic carriage of M. pneumoniae was not associated with gross or histological evidence of pulmonary disease nor with untoward effects on the overall health of hamsters.

The rhesus monkey was shown to be susceptible to infection with *M. pneumoniae* but manifestations of disease were not observed. Two of 3 infected monkeys shed the organism for at least 50 days.

As reported last year the ts-H43 mutant, which has a shutoff temperature of 37°C *in vitro*, was tested initially in 15 men who lacked serum MI antibody. During a 28 day period of intense medical surveillance none of the men developed signs or symptoms of illness. However, each of the 15 volunteers developed evidence of infection and all of the isolates recovered from the men retained the ts phenotype. Encouraged by these findings, an additional 39 volunteers were given the ts-H43 candidate vaccine strain this year in a collaborative study with Dr. Levine and Dr. Caplan. Thus, a total of 35 seronegative and 19 seropositive volunteers were given 10^6-10^7 CFU of the ts-H43 mutant by the intranasal route. Ninety-seven percent of the seronegative volunteers and 79% of the volunteers with a pre-existing MI antibody titer of 1:2 or greater were infected. Each of the isolates recovered from the volunteers retained the ts phenotype. Although febrile respiratory tract disease did not occur, bronchitis without fever developed in 4 of the 54 volunteers. Studies to determine whether fewer organisms (i.e., 10^6 CFU) can infect without producing disease are underway. In a current study volunteers given 10^4 or 10^5 CFU of ts-H43 did not develop any signs or symptoms of illness.

Of interest was the observation made in collaboration with Fernald that none of the 49 men infected by 10^6-10^7 CFU of the ts-H43 mutant developed systemic cell mediated immunity to *M. pneumoniae*. In contrast, almost all of the volunteers developed a serum antibody response and almost all of those tested developed a respiratory tract secretory antibody response. We and others have suggested that *M. pneumoniae* disease results in part from an immunologic response to infection by individuals who were sensitized to the organism during prior infection. Fernald and Clyde have suggested that cell mediated immunity may play role in the immunopathology of this disease. If this is so then the failure of ts-H43 to stimulate this type of response may offer a significant advantage to its use for immunoprophylaxis of *M. pneumoniae* disease. (Helms, Grizzard, Chanock)

Other mycoplasmas - Purification and chemical characterization of neurotoxin from *Mycoplasma neurolyticum* were extended. Variability in toxin levels of crude toxin prepared from the toxigenic strain (type A) led to more detailed examination of the relationship of growth phase of the organism to elaboration of the so-called exotoxin. Potency of whole cell broth culture toxin was directly associated with the log phase of growth and release of cell-free toxin did not appear to be associated with cold shock or lysis of cells. Centrifugation and/or ultrafiltration through 100 nm membrane filters of whole cell broth cultures revealed differences in amounts of toxin and the manipulations themselves seemed to alter potency. There would now seem to be serious questions about whether the toxin is truly an exotoxin. Column chromatography of crude toxin has successfully separated toxin from most medium protein but some loss in potency was observed.

The neutrotoxicity of crude toxin from the Type A strain and comparable materials from a non-toxigenic strain (PG-28) was studied in monkeys in collaboration with [illegible]urile. Type A toxin produced central nervous system (CNS) responses (ataxia, flaccid paralysis of limbs, and death from an ascending paralysis) with dosages of 1.5-1.7 gms/kg. A comparable CNS response was not seen in monkeys receiving the upper range of the challenge dose.

Studies on the suckling mouse cataract agent (SMCA) were carried out this year in collaboration with Dr. Fred Clark, Wistar Institute. The agent previously considered to be a slow virus was found to have the morphology and ultrastructure of a mycoplasma. Although the agent has not been grown in culture, it offers a useful model to study mycoplasma pathogenicity. Initial recovery of the agent was from a pool of rabbit ticks and this suggested the possibility that the agent might be a pathogenic form of spiroplasma. Four antisera prepared to chick embryo grown SMCA were examined in growth inhibition serological tests against *Spiroplasma citri*. Three sera had low levels of antibody to *S. citri* (2 mm inhibition) while one antiserum possessed moderate levels (4 mm) of antibody. Pre-immunization sera of each rabbit showed no activity. (Tully)

S. pneumoniae - *S. pneumoniae*, the major bacterial pathogen of otitis media, continues to be a major cause of hearing loss in early childhood. Despite the established effectiveness of the pneumoccal polysaccharide vaccine in preventing pneumococcal pneumonia in adults, preliminary studies using an octavalent pneumococcal polysaccharide preparation have indicated that the vaccine is not highly effective in preventing otitis media in infants. We have sought to take advantage of the temperature differential in the respiratory tract and have selected mutants of *S. pneumoniae* which grow vigorously at 32°C, the temperature of the upper respiratory tract, but which do not replicate efficiently at the temperature of the lower respiratory tract, 37°-38°C. Mutants of this type would be able to replicate efficiently in the nasopharynx and stimulate the production of local antibody but would not retain the capacity to produce disease in the lower respiratory tract because of their failure to grow to high titer at the restrictive temperature.

Following exposure of ts+ type I *S. pneumoniae* (PnIF strain) to NTG, 13 ts mutants were selected which were restricted in capacity to form colonies on blood agar at 38°C. While colony formation by the ts+ strain was unaffected over a broad range of temperatures (32-39°C), colony formation by ts mutants was altered or inhibited at temperatures ranging from 36°-39°C. The capacity to produce immunochemically reactive type I capsular polysaccharide was retained by ts mutants grown at permissive temperature. When administered intra-peritoneally to mice (mean core temperature 37.6$\pm$0.3°C) the ts+ strain was highly lethal ($LD_{50}=10^{0.2}$ organisms), whereas 11 of the 13 ts mutants were markedly less virulent ($LD_{50}=10^{5}-10^{7}$ organisms). In addition, mice immunized with ts mutants were resistant to subsequent lethal challenge with ts+ type I organisms. Examination of the *in vitro* growth curves of three selected ts mutants (ts-1, 3 and

4) suggest that attenuation may be related in part to altered growth rate and/or decreased survival time of ts organisms at restrictive temperatures. Following intraperitoneal inoculation of ts-1 (38°C shutoff temperature), ts-3 (36°C shutoff temperature), ts-4 (37°C shutoff temperature) or ts+ organisms, golden Syrian hamsters (mean core temperature 37.0±0.6°C) were monitored for bacteremia, serum antibody response and evidence of disease. ts+ S. pneumoniae grew readily in the blood of hamsters attaining peak concentrations in 24 to 48 hours, a time associated with the development of pathological changes, illness and death. In contrast, the three ts mutants were severely restricted in growth *in vivo*. Clearing of circulating ts organisms from the blood occurred rapidly after inoculation and was associated with the development of serum antibodies in 2-3 days. Measurable levels of antibody and resistance to lethal challenge persisted at least 4-6 weeks in the animals inoculated with the ts mutants. Despite the isolation of apparent ts+ revertants from blood of animals receiving two of the three ts mutants (ts-1 and ts-4), illness and death were not observed. Revertants of ts-3 were not detected following growth *in vivo*. (Helms, Grizzard)

Honors and Awards

Robert M. Chanock

Participated in review meetings of the Ad Hoc Study Group R & D Command, U.S. Army Department of Defense, November, 1974, March, 1975.
Member, Class Membership Committee, National Academy of Sciences, 1975
Member, Council for the Biological Sciences in Class I, University of Chicago, 1975.
Appointed to Virus Cancer Program Scientific Review Committee B, National Cancer Institute, 1974 to present
Participant, U.S.-Japan Viral Diseases Conference, Tokyo, Japan, August, 1974.
Participant, Workshop on Viral Immunology and Immunopathology, April, 1975.
Member Advisory Board *Archives of Virology*, 1975.

Robert H. Purcell

Invited speaker, 5th World Congress of Gastroenterology, Hepatitis Symposium, Mexico City, Mexico, October 13-19, 1974.
Invited speaker, Hepatitis B Symposium, Melbourne, Australia, May 2&3, 1974.
Recipient, Meritorious Service Medal, May, 1974.
Appointed, Head, Hepatitis Virus Section, March 1974.
Invited speaker, Sixth Annual Red Cross Scientific Symposium, May 8, 9, 1974.
Invited speaker, American Society for Microbiology meeting, May 6-10, 1974., Chicago, Illinois.
Invited speaker, 3rd International Congress for Virology, Madrid, Spain, September 10-17, 1975.
Invited speaker, Faculty seminar at the University of Texas Health Science Center, Dallas, Texas, November 6, 1974.
Invited speaker, Infectious Diseases Society of America, San Francisco, Calif., September 9-10, 1974.
Participant, Workshop on Viral Immunology and Immunopathology, April, 1975.

Albert Z. Kapikian

Recipient of Meritorious Service Medal of the Department of Health, Education and Welfare, U.S. Public Health Service, May 20, 1974.

Recipient of Distinguished Alumnus of the Year Award at the Baccalaureate Convocation of Queens College of the City University of New York, May 30, 1974.

Recipient of Kabakjian Award of the Armenian Students' Association of America, (A.S.A.), Inc., June 15, 1974.

Recipient of The Stitt Award at the meeting of The Association of Military Surgeons of the United States, October 29, 1974.

Invited member of World Health Organization, Food and Agriculture Organization of the United Nations Working Team on Coronaviruses in the WHO/FAO Program on Comparative Virology, 1971.

Invited member of International Coronavirus Study Group, 1972.

Invited member of the Committee on Communicable Disease Control of the American Public Health Association, 1973 - (for 12th Edition of "Control of Communicable Diseases in Man", Abram S. Benenson, Editor, Published by A.P.H.A.).

Invited speaker at meeting "What's New in Pediatric Infectious Diseases" presented by The Children's Hospital National Medical Center and the Department of Child Health and Development of the George Washington University Medical Center on May 23, 1974 in Washington, D.C. Delivered presentation on "New concepts in the Causation of Hepatitis and Gastroenteritis".

Invited speaker at NIAID workshop on "Adverse Reactions after Immunization with Inactivated Microbial Vaccines" at NIH, Bethesda, Maryland on June 10, 1974. Delivered presentation on RS Virus Immunization.

Presented paper at 8th International Congress on Electron Microscopy in Canberra, Australia on August 26, 1974. Described the detection of fastidious agents by electron microscopy.

Brian R. Murphy

Participant, Symposium on Vaccination Against Influenza, London England, April, 1975.

Michael Grizzard

Participant, annual meeting American Thoracic Society, Montreal, Canada, May, 1975.

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PHS - NIH
Individual Project Report
July, 1974 through June, 1975

Project Title: The Etiology of Acute Infectious Non-bacterial Gastroenteritis

Previous Serial Numbers: NIAID 66A, and NIAID 66H

Principal Investigators: Dr. Anthony R. Kalica
Dr. Albert Z. Kapikian
Dr. Dale H. VanKirk
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Dr. John L. Gerin (MAN Laboratory)
Dr. James Shih (MAN Laboratory)
Dr. William T. London, Dr. Amos E. Palmer (NINDS, Infectious Diseases Branch)
Dr. D. Lewis Sly (Meloy Laboratories)
Dr. Charles A. Mebus, Dr. Alfonso Torres-Medina, Mr. Norman R. Underdahl (Dept. Veterinary Sciences, University of Nebraska)

Contract Support

Project Number	Project Officer NIAID	Contractor	Contractor's Proj. Dir.	Annual Funding Level
N01 AI 32521	Dr. Chanock	Flow Labs.	Dr. Gilden	$ 491,000
N01 AI 12091	Dr. Chanock	Children's Hosp.,D.C.	Dr. Parrott	198,336

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	Total
Total:	110/12			110/12
Professional:	35/12			35/12
Others:	75/12			75/12

Project Description

Objectives - (1) To search for viruses which play an etiological role in the syndrome of acute, infectious, non-bacterial gastroenteritis of infants, children, and adults; (2) to cultivate (in vitro) the viral agents of acute infectious non-bacterial gastroenteritis; (3) to study the biophysical, immunological and epidemiological characteristics of such agents; (4) to reproduce the syndrome experimentally for the purpose of a) studying the pathophysiological and immunological responses of the host, and b) assaying the infectivity of such viral agents; (5) to develop effective immunoprophylaxis for the viruses of acute, infectious, non-bacterial gastroenteritis.

Major Findings

I. Further Studies of Norwalk-like Particles Associated with Acute Infectious Non-bacterial Gastroenteritis

(A) Summary of Previous Findings
By immune electron microscopy a 27 nm particle was visualized in a diarrheal stool filtrate (8FIIa) from an individual whose disease had been experimentally induced by administration of a Norwalk outbreak-derived stool filtrate. It was possible to use this technique to assess levels of serum antibody to the particles and to detect antibody rises following both naturally-occurring illness and illness induced by Norwalk-derived filtrates. Based on this and other evidence, it was postulated that the 27 nm particle was the etiologic agent of this particular outbreak of acute gastroenteritis. The Norwalk particle was subsequently banded by ultracentrifugation in cesium chloride and the distribution of particles was determined by immune electron microscopy. It was found to have a buoyant density of 1.38-1.41 g/cm^3, and on the basis of this and other data, it was suggested that the 27 nm particle was parvovirus-like.

The pattern of shedding of 27 nm Norwalk particles in stools during experimentally-induced gastroenteritis as determined by immune electron microscopy provided further evidence that the particle was the etiological agent of the Norwalk gastroenteritis outbreak. In addition, a similar particle, the Hawaii agent derived from a family outbreak of gastroenteritis in Hawaii was detected by immune electron microscopy.

(B) Immune Electron Microscopic Studies of Montgomery County Family Outbreak Derived Filtrates
In addition to the virus-like particles associated with the Norwalk and Hawaii outbreaks of acute epidemic non-bacterial gastroenteritis, a similar small virus-like particle was detected using materials derived from a family outbreak of gastroenteritis which occurred in Montgomery County, Maryland. In order to detect particles using immune electron microscopy (IEM), it was first necessary to treat with genetron and concentrate (10X) the stool filtrate. A serologic response was demonstrated in the "MC" particle by IEM in a naturally-occurring case of the illness in one of the family members. Additionally, paired sera from 5 volunteers who developed experimental illness following challenge with the "MC" agent were tested, and 3 developed a serologic response.

The particles derived from all three outbreaks were morphologically similar and had a similar density in cesium chloride, approximately 1.40 gm/cm^3. Paired sera from persons ill with hepatitis A or Hawaii-induced gastroenteritis did not demonstrate serum antibody increases to the MC particle; additionally persons ill with MC-induced gastroenteritis did not develop antibody responses to the Hawaii particle. However, one of 2 individuals ill with Norwalk gastroenteritis who had an antibody response to the Norwalk particle also developed an antibody response to the "MC" particle. In previous tests an individual who experienced illness with the "MC" agent also developed an antibody response to the Norwalk particle. Thus, 2 of the 3 virus-like particles from geographically-separated outbreaks of gastroenteritis appear to be related antigenically.

II. Studies of the Reovirus-like Agent Associated with Acute Infectious Non-Bacterial Gastroenteritis of Infants and Children

(A) Development of a New Complement-fixation Test for the Reovirus-like Agent of Infantile Gastroenteritis Using the Nebraska Calf Diarrhea Virus as Antigen.

A reovirus-like agent has recently been associated with acute non-bacterial gastroenteritis in infants and young children in many parts of the world. This agent appears to be the major viral etiologic agent of infantile gastroenteritis worldwide. We detected this particle in stools from infants and young children admitted to Children's Hospital of the District of Columbia with gastroenteritis. In addition, using stool filtrates as antigen we demonstrated a serologic response by complement-fixation (CF) and immune electron microscopy. Serologic responses were also detected by indirect immunofluorescence using sections of human fetal intestinal organ culture infected with the human reovirus-like agent.

Although certain stool filtrates were suitable for use in the CF test, it became apparent that the quantity of CF antigen was limited since human stools which contained enough reovirus-like particles to fix complement in our assay system were rare. We therefore attempted to develop a CF test for the serologically related Nebraska calf diarrhea virus (NCDV) in the hope that this animal virus, which grows in cell cultures and thus would be readily available, could be used as the source of CF antigen for the detection of infection with the human reovirus-like agent.

The Cody strain of NCDV received from the University of Nebraska was passaged further in our laboratory and concentrated 25 fold. This antigen was found to fix complement with convalescent sera of 3 calves which developed diarrhea following NCDV challenge; these sera were reactive at a dilution of 1:8 to 1:32 but a 1:4 dilution of the matching cord or acute phase serum was negative. Six of 8 hospitalized infants and children with gastroenteritis who shed the reovirus-like agent in stools and who developed a serological (CF) response in paired sera to the human reovirus-like stool filtrate antigen developed a seroresponse to the NCDV CF antigen. In addition, CF tests with routinely obtained paired acute or pre-illness and convalescent sera from 21 residents of Junior Village revealed that 11 of 22 selected episodes of gastroenteritis occurring during several outbreaks were associated with a significant seroresponse to a human reovirus-like stool filtrate CF antigen; in 9 of the 22 illnesses a response was detected to the NCDV antigen. Four of the seroresponses were detected only with the NCDV antigen and 6 only with the human reovirus-like CF antigen. Also, a significant seroresponse was detected in each instance when paired acute and convalescent sera from 6 reovirus-like particle positive individuals were studied by an indirect immunofluorescence technique in which NCDV was grown on coverslips of BEK and reacted with the patients' sera.

The ready availability of a cell culture grown NCDV and its use as a "substitute" CF antigen for the human reovirus-like agent should enable the serodiagnosis of many cases of disease due to the latter agent and thereby facilitate seroepidemiological studies of human infection. Also, the observation that a large proportion of individuals infected with the human reovirus-like agent develop serological (CF and indirect immunofluorescence) evidence of infection not only to the human agent but to the calf agent as well may have important implications in the immunoprophylaxis of disease caused by the human reovirus-like agent. Thus, it may be possible to immunize against the human disease utilizing the calf virus if the calf virus proves to infect humans without causing illness. If a laboratory animal can be found which responds to the NCDV with the development of disease (or infection without disease) and to the human reovirus-like agent with disease it should be possible to evaluate the protective effect of prior NCDV infection upon disease caused by the human reovirus-like agent.

(B) Antigenic Relationships Among the Human Reovirus-like Agent, NCDV and Epizootic Diarrhea of Infant Mice (EDIM) Virus

We also utilized the CF technique to determine if an antigenic relationship existed between the NCDV and the EDIM viruses and to further study the already known serological relationship between these animal viruses and the human reovirus-like agent. All 3 antigens reacted with an immune NCDV rabbit serum and an EDIM virus immune serum from mice whereas only the NCDV and human reovirus-like agent reacted with an immune guinea pig serum raised to the human reovirus-like agent. The EDIM virus failed to react with a convalescent NCDV calf serum while the human reovirus-like agent failed to react with this serum on several occasions but in one test reacted to low titer. Two children who in previous tests had demonstrated serological (CF) evidence of infection with the human reovirus-like agent and NCDV and had developed relatively high titers of antibody to each in convalescent sera, were selected for a comparison of antibody responses to the NCDV, the human reovirus-like agent, and the EDIM virus. Both individuals again developed serological evidence of infection with the human reovirus-like agent and NCDV, but only one responded to the EDIM virus.

Thus, the NCDV and EDIM virus are serologically related and the human reovirus-like agent, which has previously been shown to be related to both of these viruses, is probably more closely related to the calf agent than it is to the mouse agent. Both the EDIM virus immune serum from mice and the NCDV immune rabbit serum gave broader CF antibody responses than the human reovirus-like agent immune guinea pig serum as they reacted with all three reovirus-like agents. Possibly these differences in CF reactivity might offer a means of differentiating the three reovirus-like agents.

(C) Relationship of the SA-11 and "O" Reovirus-like Agents to the Human Reovirus-like Agent, NCDV and EDIM Viruses

The reovirus-like SA-11 agent (originally isolated from a rectal swab from a healthy vervet monkey) and the "O" agent (originally isolated from intestinal washings of cattle and sheep) each react significantly by CF with hyperimmune sera to the human reovirus-like agent, the NCDV and the EDIM virus. It thus appears that these 5 reovirus-like agents are antigenically related. Hyperimmune antisera to the SA-11 and "O" agents are currently being prepared.

(D) Relationship of NCDV to Reovirus Types 1, 2, 3, and Twenty Orbiviruses by the CF Technique

In reciprocal CF tests the NCDV was not related to the 3 human reovirus serotypes. The lack of a relationship of the NCDV and reovirus types 1 and 3 had been shown previously by others using other methods.

The NCDV antigen did not react with a 1:2 dilution of two different polyvalent ascitic fluids from immunized mice which contained CF antibody to the following fourteen orbiviruses: Polyam; I 68886; IG 15534; Corriparta; Eth Ar 1846-64; Eubenangee; Dak ArB 1327; D'Aguilar; Kemerovo; Chenuda; Mono Lake; Wad Medani; Tribec; and Huacho. However, the NCDV antigen did react with a 1:2 dilution but not significantly with a 1:4 dilution of polyvalent ascitic fluid from immunized mice which contained antibody to bluetongue, epizootic hemorrhagic disease of deer (FHD), IbAR 22619, Changuinola, Irituia, and Colorado tick fever (CTF) viruses. Individual ascitic fluids were available for the Changuinola, EHD and CTF antigens. CF tests with these fluids revealed that the NCDV antigen reacted with a 1:2 dilution but not significantly with a 1:4 dilution of Changuinola ascitic fluid. A significant reaction did not occur with the EHD and CTF fluids.

(E) Biophysical Studies of the NCDV CF Antigen

2 ml of 25X concentrated NCDV was centrifuged at 80,600g (average) for 1 hr. at 4°C. The supernatant fluid and resuspended pellet were each studied for CF activity with a 1:8 dilution of NCDV convalescent calf serum. The supernatant fluid failed to react with this serum whereas the resuspended pellet reacted to an antigen dilution of 1:8.

In additional studies, 25X concentrated NCDV was doubly banded in cesium chloride. Starting material for the second banding consisted of 3 fractions from the first banding which contained the largest number of NCDV particles detected by direct electron microscopic examination of negatively stained fractions. Each of 23 fractions from the second banding was examined for CF activity with a convalescent calf serum. Peak CF activity of 16 units coincided with the peak fraction, number 8 (density 1.373), which contained 70.2% of the NCDV particles by electron microscopy. Fractions 7 (density 1.386), 9 (density 1.364) and 10 (density 1.353) had CF activity of 1, 4 and 1 units respectively; in addition, such fractions contained 2.3%, 22.6% and 3.1% respectively, of the total 1596 particles counted. The remaining nineteen fractions each failed to react in the CF test. In a repeat CF test fraction 8 contained 16 units and fraction 9 contained 8 units of antigen whereas the remaining twenty-one fractions each failed to react significantly.

Thus, the CF activity of the NCDV antigen was closely associated with the NCDV virion. In addition, the density of NCDV found in this study was compatible with that reported previously.

(F) Prevalence of CF Antibody to NCDV and the Human Reovirus-like Agent in Sera from Infants and Children

Sera obtained within 6 days of admission to Junior Village from 1954 through 1969 from selected infants and children aged 6-60 months were tested for the prevalence of CF antibody (at a titer of 1:4) to both the NCDV and human reovirus-like antigens. This survey showed that antibody to both these agents was acquired at an early age. Of 70 infants, aged 6-12 months, whose sera were tested satisfactorily against both antigens, 31 (46%) possessed CF antibody to the NCDV and 41 (59%) to the human reovirus-like agent. A comparison of the prevalence of CF antibody in the sera obtained from the 6-12 month-age-group from 1954 to the early 1960's with that in sera obtained from other infants of the same age group from the early 1960's to 1969 revealed that the prevalence of antibody in the former group of sera was significantly greater to either antigen: 70% vs 38% (P 0.01, X^2 test) for the human reovirus-like agent and 57% vs 27% (P 0.02 X^2 test) for NCDV. This preliminary serological survey suggests that the infection rate with the human agent varies over different time periods. A strong positive association was found in the prevalence of antibody to the 2 antigens; concordance was found in 82 (75%) of 110 sera tested (V= +0.486 Kendall coefficient of association; X^2=25.9). However, as observed in the detection of seroresponses, the human reovirus-like agent appeared to be more efficient in general, than the NCDV in detecting antibody to the human agent.

Over 90% of the infants and children possessed antibody to the human reovirus-like agent by 36 months of age; over 80% had antibody to NCDV. This pattern of rapid acquisition of antibody to the reovirus-like agent is comparable to that observed for the respiratory syncytial and parainfluenza type 3 viruses and provides convincing serological confirmation of epidemiological data which indicates that infection with the human reovirus-like agent occurs commonly during infancy. Thus, this young age group which is at high risk of developing severe diarrheal disease would be a prime candidate for immunoprophylaxis when, and if, a safe effective vaccine is developed for disease caused by the human reovirus-like agent.

(G) Studies on the Etiology of Non-Bacterial Gastroenteritis Among Infants and Children Admitted to Children's Hospital (D.C.)

In January, 1974 we expanded our gastroenteritis studies which had been limited to outbreaks in schools, institutions, the military, and families to include infants and young children admitted to the Children's Hospital of the District of Columbia with acute gastroenteritis in an attempt to detect the etiologic agent(s) of acute gastroenteritis in this age group. We then extended these studies to include controls and more recently further expanded them to include contacts (parents, siblings, etc.) of patients admitted to the hospital. Study of contacts was initiated to determine whether the reovirus-like agent was capable of infecting the older members of the patient's family; such information might help to elucidate the mode of transmission of this common agent.

One hundred ten patients who had gastroenteritis and were hospitalized from January, 1974 through February, 1975 were studied by electron microscopy for the presence of viruses in filtrates made from their stools. Although conventional electron microscopy was utilized occasionally, the technique of immune electron microscopy was employed in all instances in order to detect small parvovirus-like particles which would not normally have been detected by conventional electron microscopy (EM). The reovirus-like agent is normally detectable by either method. Preliminary analysis revealed that 52 (47%) of the 110 infants and children with gastroenteritis were shedding the reovirus-like agent in stools. Peak virus shedding occurred in the "winter" months, --January, February and December, 1974 and January, February, 1975 when the reovirus-like agent was detected in stools of 39 (72%) of 53 infants and children. Virus was not detected from June, 1974 throgh October, 1974. Paired sera from 40 of the 52 virus-positive individuals were available, and 31 of the 40 demonstrated serologic evidence of infection with the human reovirus-like agent and/or NCDV. Nine individuals shed the virus but did not develop a seroresponse by the CF technique. The efficiency of virus detection by EM among hospitalized individuals was striking as serologic evidence of infection without virus shedding occurred only rarely. Preliminary studies of certain adult family contacts of hospitalized infants and children revealed that 11 mothers and one father developed serologic evidence of infection with the human reovirus-like agent and/or the NCDV. Only one of these contacts had gastroenteritis with diarrhea; this illness developed 6 days following hospitalization of the index case. In addition, a resident physician at Children's hospital developed severe diarrhea and serologic evidence of infection to the NCDV. Stools were available from 6 of these adults and none were found to contain the reovirus-like agent. Additional studies are in progress in an attempt to detect the reovirus-like agent in these adult stools. The occurrence of infection in certain adult contacts may have important epidemiologic implications especially since a few of the contacts had pre-existing CF antibody. It appears that adults can be infected or reinfected with the reovirus-like agent and thus may be a source of virus for transmission to infants who are normally not in contact with other infants.

(H) Inoculation of Volunteers with the Human Reovirus-like Agent
Two adult volunteers who possessed pre-existing (CF and indirect immunofluorescence) serum antibody to the human reovirus-like agent were studied in the Clinical Center. A safety tested inoculum, consisting of 1 ml. of a 1:10 dilution of an approximate 2% stool filtrate prepared from a stool obtained from a 13 month-old child (D) hospitalized with gastroenteritis, was administered orally to each volunteer following further dilution in 30 ml of tap water. Each volunteer received sodium bicarbonate orally immediately prior to administration of the virus containing solution. Neither volunteer developed definite illness following challenge. However, one volunteer developed abdominal cramps about 74 hours after challenge. This volunteer also had the onset of her menstrual period on the same day as the cramps occurred. The patient related that cramps had not occurred with her periods in the past and that this would be the first time of such an occurrence. Tests with paired pre- and post-challenge sera, revealed that the volunteer who developed cramps developed serologic (CF) evidence of infection with the human reovirus-like agent whereas the other volunteer did not. Reovirus-like particles were not detected in stool filtrates prepared from stools of the volunteers. Additional studies of the stools of the volunteer who developed a seroconversion are in progress.

(I) Studies in Animals with the Human Reovirus-like Agent
A variety of animals were inoculated with a 2% human stool filtrate containing the reovirus-like agent. No signs of illness developed in the following animals inoculated by the indicated route: suckling mice (oral intracranial, subcutaneous), adult mice (intracranial), guinea pigs (intraperitoneal), and rabbits (corneal). Antibody to the human reovirus-like agent was not detected by CF or indirect immunofluorescence in pooled sera from suckling mice 30 days following oral inoculation, and virus could not be detected by electron microscopy in intestinal homogenates made from suckling mice 7 days following inoculation.

In a collaborative study with the Department of Veterinary Science, University of Nebraska, one to 4 day old gnotobiotic piglets were inoculated orally with a human stool filtrate containing the reovirus-like agent. Diarrhea developed in the piglets 30-155 hrs. after inoculation with filtrate from patient Fs, and was reproduced for 5 serial passages in one sequence and for 3 passages in another. Another stool filtrate from patient D was also demonstrated to be infectious for gnotobiotic piglets. Nineteen of 21 inoculated piglets developed diarrhea after inoculation with the "Fs" strain; reovirus-like particles were observed in intestinal contents and/or fecal samples from 17 animals with illness and from 2 inoculated piglets that did not develop diarrhea. In 5 piglets studied by EM thus far, the largest number of virus particles was shed one day prior to or on the day of onset of diarrhea. Serum antibody responses to the human reovirus-like agent were observed in the 7 inoculated animals tested by indirect immunofluorescence.

Caesarean-derived colostrum-deprived gnotobiotic calves have also been studied in collaboration with the University of Nebraska. Four calves were given the "Fs" or "D" strain of human reovirus-like agent in the form of a

2% human stool filtrate; inoculation was performed by intraduodenal injection at laparotomy. None of the animals which received virus-containing human stool filtrate developed illness, but each developed evidence of infection as indicated by electron microscopic detection of reovirus-like particles in stools. Two additional colostrum-deprived calves received 2% bacteria-free calf stool filtrates containing the human virus derived from first passage of the agent in calves. Both introduodenally-inoculated animals developed self-limited diarrhea approximately 27 to 29 hours following inoculation.

Rhesus monkeys were also studied (in collaboration with Drs. W.T. London, D.L. Sly and A.E. Palmer for susceptibility to illness with the human reovirus-like agent. Junvenile monkeys were studied initially, and it appeared that infection occurred in the absence of illness since 3 of 4 inoculated animals developed serum antibody to the virus. Subsequently, 8-day-old infant rhesus monkeys, which were allowed to deliver naturally and were breast-fed during the first 8 days of life, were inoculated with the virus. Although no illness developed, infection occurred as evidenced by shedding of small numbers of reovirus-like particle in stools and seroresponses in 2 of 4 animals. Subsequently, Caesarean-delivered rhesus monkeys were inoculated on the first day of life before the animals received breast milk. Diarrhea developed in 5 of 7 animals. Incubation period ranged from 2-5 days; virus particles were detected in stools in association with illness, and virus shedding lasted 1-3 days. Virus derived from monkeys that developed illness following inoculation was infectious for other monkeys but did not induce diarrhea which could be associated with virus shedding. Virus was also detected in tissues of the small intestine of an acutely ill monkey using immunofluorescence and ultrathin section electron microscopy. Serum antibody responses were demonstrated in two of the ill animals by complement-fixation and/or immunofluorescence.

(J) Further Studies with the Human Reovirus-like Agent Cultivated In Vitro Using Human Fetal Intestinal Organ Culture

The human reovirus-like agent was passaged four times using the organ culture system, but viral growth did not progress to involve more than small foci and single cells of the explants. Only 3 of 15 virus particle containing stool filtrates proved to be infectious for the organ culture system. Paired sera from 23 patients with gastroenteritis from D.C. Children's Hospital were examined for an antibody response using frozen sections of infected organ cultures as a source of antigen for the indirect immunofluorescence test. Twelve of these patients had reovirus-like particles in their stools as detected by electron microscopy, and 11 of the 12 developed an antibody response to the virus. Eleven of the 23 patients did not have reovirus-like particles detected in stools, and only 2 developed an antibody response to the virus by this technique.

In an attempt to determine the age at which infection occurs and what proportion of infants and young children become infected with this virus, 70 serum samples obtained from infants and children on admission to D.C. Children's Hospital were studied; the patients did not have gastroenteric illness and ranged in age between 7 and 59 months. Sera were tested by indirect immunofluorescence at a 1:10 dilution using sections of infected organ culture. A significant trend of increasing relative frequency of antibody with increasing age was observed. Nine of 15 infants between 7 and 11 months of age had antibody to the human reovirus-like agent, and in the second year of life 10 of 13 children had demonstrable antibody. During the third, fourth and fifth years of life antibody was detected in 40 of 42 children tested.

Fetal rhesus monkey intestine, fetal pig intestine, and fetal bovine intestine were tested for susceptibility to infection with the human reovirus-like agent. A few infected cells were detected in monkey and pig tissues.

(K) Attempts to Cultivate the Human Reovirus-like Agent with Cell Cultures

Continued efforts to grow the human reovirus-like agent in cell culture have utilized primarily WI-38, human embryonic kidney, rhesus monkey kidney, bovine embryonic kidney, porcine embryonic kidney, and to a lesser extent other selected cell cultures. A variety of serum-free media was used in these attempts. Twenty-six reovirus-like particle positive stool filtrates from patients with gastroenteritis were inoculated into cell cultures. A passage series derived from one of these filtrates has exhibitited a granular cytopathic effect and this putative strain derived from patient "Fs" is now in its 10th passage. Virus was detected in cultures from this passage series by both immunofluorescence and electron microscopy, and confirmation of these findings and tests to establish specificity are underway to rule out the possibility of laboratory contamination with one of the related animal viruses.

Other animal viruses related to the human reovirus-like agent have been cultured in the laboratory in order to prepare reagents for antigenic analysis of the group. These viruses include the vervet monkey SA-11 virus, "O" agent from sheep and cattle, and the Nebraska Calf Diarrhea Virus. Attempts have been made to cultivate EDIM virus *in vitro* but these have failed.

(L) Buoyant Density in Cesium Chloride of the Human Reovirus-like Agent

In order to further characterize the human reovirus-like agent we determined the buoyant density in cesium chloride of 3 different filtrates made from stools obtained from children with gastroenteritis. The first filtrate studied was made from a stool from patient D. Visual examination of the tube following ultracentrifugation revealed 4 light scattering bands: one broad band between 1.1-1.2g/cm^3 and three discrete ones at about 1.36, slightly greater than 1.40, and 1.43 gm/cm^3. Examination of each of the gradient fractions by IEM revealed the presence of at least one reovirus-like particle in 9 of the 22 fractions; they occurred as single particles, doublets and small or large aggregates. As a quantitative estimate of the distribution of reovirus-like particles in the density gradient, the number of particles in each of the 9 positive fractions was counted and the number found in each fraction expressed as a percentage of the total number of particles. A total of 1857 particles were counted in the 9 positive fractions. Particles (which were predominantly "full") were distributed in an approximately normal distribution around the peak fraction which had a density of 1.35.

In another experiment using a filtrate derived from patient S the density of particles and CF antigen activity was determined. The peak number of "full" particles was found at a density of 1.374 whereas the peak number of "empty" particles was observed at a density of 1.286. Peak CF activity was present at these 2 densities. In other studies by differential centrifugation, it was also found that CF activity appeared to be associated with the human reovirus-like agent virion.

A third filtrate from a stool from patient P was also studied. Aliquots from four contiguous fractions of a cesium chloride density gradient were examined directly by electron microscopy and the peak fraction was found to be at a density of 1.354.

Significance to Biomedical Research and the Program of the Institute

Acute infectious non-bacterial gastroenteritis is a common infectious disease which affects a broad segment of the population and was the second most common disease observed in a 10 year family study. Ultimate goals of prevention and therapy have been furthered by (1) the detection and preliminary characterization of etiologic agents of this disease, (2) the development of serologic assay systems by which the epidemiologic importance of the disease can be determined, (3) the ability to cultivate in vitro the reovirus-like agent and (4) the ability to induce illness in experimental animals with the reovirus-like agent. The progress in studying the reovirus-like agent of gastroenteritis in infants and young children represents a significant step in understanding this disease of world-wide importance.

Proposed Course of the Project

Intensive study of etiologic agent(s) from outbreaks of acute, infectious non-bacterial gastroenteritis and from clinical specimens from Children's Hospital will continue. In vitro propagation of the agent(s) will be studied further using a variety of cell culture systems. Further biophysical purification and characterization of these agents, development of more sensitive serologic assay systems, and methods for rapid diagnosis of this disease will be major goals. Studies of the pathogenesis of disease in animal models will also be pursued. Development of a vaccine to prevent disease due to the human reovirus-like agent will be a major goal of this project.

Keyword Descriptors

Acute infectious non-bacterial gastroenteritis
Viral gastroenteritis
Viral diarrhea
Infantile gastroenteritis
Reovirus-like agents
Parvovirus-like agents
Norwalk agent

Immune electron microscopy
Animal models
Epidemiology

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1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Study of Non-bacterial Respiratory Disease in Children

Previous Serial No.: NIAID 69A

Principal Investigator: Dr. Robert M. Chanock

Other Investigators: Dr. T.J. Schnitzer
Dr. L.S. Richardson

Cooperating Units: Dr. R.H. Parrott, Children's Hospital Research Foundation of Washington, D.C.

Contract Support:

Contract Number	Project Off. (NIAID)	Contractor	Contractor's Project. Dir.	Annual Funding Level
NO1 AI 32521	Dr. Chanock	Flow Labs.	Dr. Gilden	$ 490,000
NO1 AI 12091	Dr. Chanock	Children's Hosp. D.C.	DR. Parrott	198,336

Man Years;	NIAID Lab Staff	Other NIH Personnel	Guest Workers	Total
Total:	12/12			12/12
Professional:	6/12			6/12
Other:	6/12			6/12

Project Description

Objectives: To delineate the epidemiology and pathogenesis of viruses which cause respiratory disease in early life

Influenza A and B Virus Infection and Illness of Infants and Young Children; The influenza virus experience of 27,000 infants and young children seen at Children's Hospital of D.C. during the past 18 years was analyzed. This was done in an attempt to assess the impact of the type A and type B viruses in early life and to define temporal and age related patterns of disease. The overall recovery of influenza A virus during the 1957-1968 H2N2 era was very low (overall 0.5%) while during the 1968-1975 H3N2 era the frequency of isolation was 6 fold higher (3.1%). In part, this probably reflects the almost yearly pattern of the H3N2 subtype versus the bi- or tri-yearly epidemics produced by the H2N2 viruses. The greater insensitivity of tissue cultures for revoery of H2N2 viruses was probably also a major factor in this difference. Influenza A virus was most strongly associated with croup (acute laryngotracheobronchitis).

During the H2N2 era 7.7% of croup illnesses could be associated with influenza A virus infection while from 1968-1975 (i.e., the H3N2 era) 24% of croup patients were infected with type A virus. The contribution of type A virus to the other respiratory tract diseases was less; for example, the estimate for pneumonia was 3.5% during 1957-1968 and 4.6% during 1968-1975. Influenza A croup appeared to be a disease primarily of the first year of life. During the peak month of influenza A virus activity in the community this virus was associated with 70% of the croup illnesses which required admission to the hospital; the comparable figure for all respiratory disease admissions to the hospital was 36%. Of interest was the importance of influenza B virus in the croup syndrome during the peak month of type B virus activity--25% of croup patients were infected with this virus. These observations provide support for the view that infants and young children should be immunized against the influenza viruses.

Keyword Descriptors

Non-bacterial respiratory disease
Respiratory viruses
Epidemiology

Publications

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(1) Infectious Diseases
(2) Epidemiology
(3) Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Etiology of Acute Respiratory Diseases in Adults

Previous Serial Nos.: NIAID 69C, 70B, 72A

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigators: Dr. Robert M. Chanock
Dr. Anthony R. Kalica

Descrption

This project has been discontinued. All work has been reported in other projects.

Serial No. Z01 AI 00024-15 LID
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Laboratory Studies of Myxoviruses, Paramyxoviruses and Respiratory Syncytial Virus

Previous Serial No.: NIAID 70A

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Total:	96/12			96/12
Professional:	48/12			48/12
Other:	48/12			48/12

Project Description

Objectives: (1) To produce conditional lethal temperature-sensitive (ts) mutants of myxoviruses, paramyxoviruses and RS virus for possible use in live attenuated vaccines; (2) to study the genetic basis for attenuation shown by temperature sensitive mutants; (3) to define the relative importance of the various host defense mechanisms in resistance to myxovirus and paramyxovirus infection and disease.

Major Findings

(A) Respiratory Syncytial Virus
Efforts to further characterize the 7 stable ts mutants of RS virus were directed toward: (1) a more precise definition of factors determining the growth capacity and genetic stability of ts-1 at restrictive temperatures (37°-39°C) and (2) a more detailed examination of the growth, antigen production, and RNA synthesis of each of the ts mutants under restrictive conditions.

LI
LSD
RML
LBV
LMI
LPD

1. Further characterization of the ts-1 mutants - Studies were performed to define those factors which determined the growth of ts-1 virus in vitro at restrictive temperatures and the emergence of genetically altered virus under these conditions. Growth of the ts-1 mutant occurred at all restrictive temperatures; the level of replication, however, was inversely related to the incubation temperature and was significantly reduced compared either to peak wild type RS virus titer at the same temperature or the peak titer attained by the ts-1 mutant at permissive temperature. During these studies it became apparent that viral growth under restrictive conditions was a function not only of the incubation temperature but also of inoculum size. Titers attained at 6 days after inoculation of replicate cell cultures with different amounts of ts-1 virus were found to be directly and linearly related to the amount of input virus; this relationship was observed at each of the restrictive temperatures tested but was not seen at permissive temperature. At the permissive temperature of 32°C, lowering the multiplicity of infection (MOI) delayed the onset, but not the magnitude of the peak viral titer. At restrictive temperatures, however, maximal level of replication was achieved by the third day post-inoculation and this level was then maintained for the duration of the experiment, i.e., an additional 3 days. The plateau titer attained was directly related to the size of the input inoculum. These data suggested that suppression of viral growth observed at restrictive temperature was a consequence of two factors: (1) a decrease in production and release of infectious virus by infected cells and (2) prevention of spread of infection within the culture. These inferences were confirmed by appropriate experiments. The virus yield per infected cell was found to be decreased 30-50 fold at 37°C and 38°C and was suppressed 1000 fold or more at 39°C compared to production of infectious virus at 32°C. At restrictive temperatures the quantity of virus released by infected cells was less than that observed at permissive temperature. Using fluorescent focus and infectious center assays it was shown that viral spread was retarded at 37°C and totally inhibited at higher incubation temperatures, probably as a consequence of decreased production and release of virus. However, ts-1 was able to initiate infection equally well at all temperatures and the mutant persisted in infected cells at restrictive temperatures in a manner which permitted expression of some viral functions without destruction of these infected cells and which allowed full expression of all viral functions, with the resultant production of infectious progeny, following shift down to permissive temperature.

In recent vaccine trials of the ts-1 mutant in infants and young children a small proportion of the virus recovered exhibited a partial or complete loss of temperature sensitivity, i.e., change from ts to ts+. Subsequently, the emergence of ts+ virus in cell cultures incubated at restrictive temperature was also noted. Genetic alteration in vitro, however, occurred irregularly and unpredictably. This in vitro phenomenon was investigated further after the importance of multiplicity of infection in growth of ts-1 at restrictive temperature was appreciated. As anticipated, the occurrence of genetic alteration in the ts-1 mutant was a direct function of input multiplicity. At the restrictive temperatures of 37°C and 38°C, 90-100% of cultures infected at an MOI of 0.1 pfu per cell produced ts+ virus, whereas only 25% of cultures infected at an MOI of .01 pfu per cell yielded such altered virus. Importantly, altered virus was never detected in the growth yield of virus grown at permissive temperature, although the quantity of virus produced was considered greater than that generated under restrictive conditions. Emergence of virus with altered temperature sensitivity was more frequent in cultures incubated at 37°C than in cells kept at 38°C. Genetic change in ts-1 thus appeared to be favored by those conditions which facilitated virus growth--high multiplicity of infection and the low restrictive temperature of 37°C. This suggested that genetic alteration of ts-1 was a random event which occurred with a fixed frequency during viral replication. The fact that ts+ virus did not develop at 32°C, although virus growth was optimal at this temperature, suggests that elevated temperature itself may play a role in increasing the frequency of mutation, possibly through an action on an RNA dependent polymerase.

2. Growth, antigen production and RNA synthesis by other ts mutants - Further examination of the RNA synthesis, antigen production and growth of the ts mutants was undertaken during the past year. Previous work had indicated that only ts-2 of the 7 stable RS mutants produced viral-specific RNA at restrictive temperature. However, when a high MOI was employed ts-7 was shown to be also RNA+ at 39°C. None of the mutants in the third complementation group (ts-1, 3, 4, 5, 6) produced any detectable viral specific RNA at 39°C. Methods for detecting RSV specific RNA are insensitive and the possibility exists that ts mutants in this complementation group produce amounts of viral specific RNA that are below the level of current methods of detection. The RNA polymerase activity of each of the 7 mutants had previously been shown to be functionally intact at restrictive temperature.

Each of the mutants except ts-4 had been shown to produce viral specific antigen(s) at restrictive temperature (39°C). Recent studies employing a high MOI of ts-4 indicated that a small percentage (<1%) of cells inoculated with this mutant and incubated at 39°C developed RS virus specific antigen(s). Whether such antigen-positive cells contained altered virus is not known.

Examination of the growth of the ts-2 and ts-7 mutants at permissive and restrictive temperaures was undertaken as described above for ts-1. Ts-2 had been shown to grow with less restriction at elevated temperatures than the other mutants; at 39°C this mutant exhibited only a 90% reduction in growth. This was confirmed for cultures infected with high inputs of ts-2. However, at lower inputs, results were similar to those reported for ts-1. Peak viral titers in cultures incubated at restrictive temperature were both temperature and input dependent and, once reached, were maintained. This suggested that inhibition of viral spread was an important factor in limitation of growth at high temperature and this was confirmed by fluorescent focus and infectious center assays as described for ts-1. Examination of growth of ts-7 under similar conditions demonstrated a different pattern from that seen with either ts-1 or ts-2. Viral growth at 37°C approximated that seen at 32°C; growth at 38°C was depressed relative to the lower temperatures, but continued to increase during the entire course of the 6 day growth study suggesting the occurrence of continued viral spread. This was documented by fluorescent focus and infectious center assay. In cultures incubated at 39°C, viral growth was not sustained over the course of the experiment. Cells initially capable of giving rise to infectious centers did not maintain this potential after further incubation at 39°C. Spread of virus was not detected.

3. Introduction of additional genetic lesions into the ts-1 mutant of RS virus - Evaluation of the ts-1 mutant in young vaccinees had shown that it was not acceptable as a vaccine strain due to its low level of residual virulence and the emergence of genetically altered virus during growth *in vivo*. In a continuing effort to produce a vaccine for RS virus, as reported last year, we attempted to produce a more defective virus than ts-1 by introduction of further genetic lesions into the ts-1 virus using nitrosoguanidine (NTG) as the mutagen. The basis of selection for a more defective virus was loss of ability to produce plaques at 36°C; the ts-1 virus produces plaques at 36° but is markedly or completely restricted in this function at 37°C.

Plaques were harvested from bovine kidney (BK) cultures which had been inoculated 6 days earlier with ts-1 virus which had been treated with 15 μg/ml of NTG. Plaque progeny were passaged in roller tube cultures in order to prepare a working suspension of virus for characterization. Each of the plaque populations was tested for ability to produce plaques at 36°C. A total of 427 plaque populations were tested in this manner. Only 2 plaque populations (#230 and 266) exhibited an alteration in plaquing efficiency at 36°C; both #230 and 266 were at least 100 fold less efficient in plaque production at 36°C than at 32°C (a permissive temperature). These 2 clones were purified by 2 additional plaque passages in bovine kidney cells. A total of 13 subclones (3 from the original 230 clone and 15 from the 266 clone) which exhibited a 100 fold or greater reduction in plaque formation at 36°C were than characterized in greater detail.

In an attempt to differentiate among these 18 subclones we tested their ability to replicate at the restrictive temperatures of 36°C, 37°C and 38°C. Each subclone was inoculated into HEp-2 tube cultures and incubated for 5-6 days at a permissive (32°C) or restrictive (36°C, 37°C or 38°C) temperature. Total yield of virus was assayed at 32°C (a permissive temperature) and the growth of genetically altered virus was studied by plaque assays performed at the restrictive temperatures of 37°C and 38°C.

When HEp-2 cells were inoculated at a multiplicity of infection (MOI) of 0.1 plaque forming unit/cell or greater, growth of each of the sub-clones was 10 to 100 fold lower than ts-1 at each of the restrictive temperatures. In addition, genetically altered virus able to form plaques at 37°C was detected occasionally in the growth yield under these conditions, whereas ts-1 regularly produced altered virus which induced plaques at both 37°C and 38°C. The results of these tests allowed us to select the subclones which were most defective in growth at restrictive temperatures and which were most stable genetically.

The 8 most promising subclones, 3 from clone 230 and 5 from clone 266 were further evaluated by inoculating a high input (MOI of 1) into HEp-2 tube cultures. The growth of the 8 subclones was again 10-100 fold lower than ts-1 at the restrictive temperatures, and genetically altered progeny were detected less frequently than with ts-1. In addition, the subclones derived from clone 230 appeared to be relatively uniform and could be differentiated from subclones derived from clone 266. Four of the subclones were examined in greater detail for ability to replicate at restrictive temperature and each was found to be more defective than ts-1.

The nature of the additional ts lesions induced in the ts-1 mutant by treatment with NTG was investigated by the monolayer culture complementation test described previously. Two subclones, 230-13-5 and 266-6-5, from NTG treated ts-1 did not complement ts-1, as expected, but they did complement ts-2 and ts-7 which belong to the other 2 complementation groups of RS ts mutants. Thus, the new lesions induced in ts-1 by NTG were not in complementation groups 2 and 3.

230-13-5 and 266-6-5 were inoculated into hamsters to determine their ability to grow, to elicit an antibody response, and to protect against challenge with the wild type RS virus. Limited growth of the 230-13-5 subclone was detected in the nasal turbinates, but virus was not recovered from the lungs. Growth of 230-12-15 in the turbinates was approximately 10 fold lower than that seen with ts-1. Evidence of viral growth in the hamster was not detected with the second subclone (266-6-5). The antibody response of the hamsters and their response to challenge with virulent wild type RS virus is currently under study. Studies with these viruses in newborn ferrets are also underway since ferrets have been shown by Porter and Prince to be a more permissive host for RS virus than are hamsters.

4. Attempts to establish persistent infection in vitro with RS virus - Simpson recently reported that both wild type and ts RS virus established a persistent infection in cell culture and that DNA intermediates complementary to viral RNA were present in cells which exhibited no other evidence of infection. We have attempted to confirm this important finding with the ts-1 mutant. HEp-2, human embryonic kidney (HEK), human embryonic lung (HEL) and bovine embyronic kidney (BEK) cells were exposed to the ts-1 mutant at an MOI of 0.1 or 0.001 and the cultures incubated at 38° or 39°C, temperatures which are restrictive for ts-1. The HEK and HEL cells were destroyed by the virus within 1-3 passages. Similarly the low and high MOI 38°C HEp-2 cells and high MOI 38°C HEp-2 cells underwent complete cytolysis. The low MOI BEK (38°C and 39°C) was well as the HEp-2 (39°C) cultures grew without cytopathathic effect. These cells were studied for evidence of viral activity by assay for infectious virus in supernatant fluid, by infectious center assay, by co-cultivation and by indirect immunofluorescence. Viral activity was not detected in the 39°C HEp-2 and BEK cells by these methods. In contrast, evidence of viral persistence was detected in the 38°C BEK cells which have been successively kept in serial cultivations for 3 months. The proportion of cells with RS antigen or which registered as infectious centers was very small (<1%).

Virus recovered from the 38°C BEK cells retained the ts phenotype. An effort to initiate a persistent infection in Vero cells with the Schwarz strain of measles virus was successful and like the ts-1 persistent infection only a small proportion (<1%) of the cells contain immunofluorescent antigen.

(B) Influenza Viruses

1. Isolation and characterization of temperature-sensitive mutants of influenza A virus - The purpose of further isolation and characterization of ts mutants of influenza A is to (1) derive a series of recombinants which have a single lesion or cluster of lesions on one of the functional portions of the influenza A virus genome with the aim of producing ts viruses with lesions specific for each of the viral genes; (2) derive separate mutants which have a ts defect in the same segment of the influenza A virus genome but which differ with regard to temperature sensitivity; (3) further evaluate ts viruses in seronegative volunteers to explore more fully the relationship between attenuation, site of genetic lesion and level of temperature sensitivity. Such single lesioned mutants could also serve as genetic probes for characterization of the lesion or lesions transferred from an older ts vaccine to a newly emerged influenza strain possessing new surface glycoprotein antigens in order to verify that a putative ts recombinant virus possesses only the desired ts lesions of its attenuated parental strain.

As previously reported we have generated 2 sets of ts mutants with the H3N2 surface antigen (i) 4 ts influenza viruses (ts-1[A], ts-1[E], ts-1[H], ts-2[C]) derived by recombination of a ts mutant of H2N2 with wild type H3N2 (ii) a series of 9 different clones of ts viruses which were isolated from a directly mutagenized stock of H3N2 virus. In addition, 3 ts recombinants were produced by mating H3N2 ts-1[E] and wild type H0N1 influenza A viruses (R1, R8 and R11).

Using the plate complementation-recombination test previously described we have identified 9 single lesioned mutants and assigned them to 7 complementation groups. Since the mutants were derived as the source of ts lesions which could be transferred into new epidemic variants, the lesions which are of interest to us must of necessity not be in the portion of the genome which encodes the 2 surface glycoproteins (the hemagglutinin and the neuraminidase). Ts-1[A], ts-1[E], ts-1[H] and ts-2[C] were derived by recombination and selection for ts recombinants bearing a different ($H3_{68}$) hemagglutinin than the parental ts-1 and ts-2 (H2) strains. Therefore, the lesions in these mutants were not on the hemagglutinin gene. In addition, the second transfer of the ts-1[A] and ts-1[E] lesions into a $H3_{72}N2_{72}$ glycoprotein coat demonstrated that the ts lesions of these 2 viruses did not affect the neuraminidase gene. In order to more rapidly ascertain whether the single lesioned H3N2 ts mutants (304, 315, 422, 463, 454, and 464) had lesions in genes coding for the surface glycoproteins we adapted the marker rescue test developed by Sugiura. Each H3N2 ts mutant was recombined with the H0N1 wild type strain. Antisera to the H0N1 virus was then added and the mixture plated at the restrictive temperature of 39°C. Plaques which developed at this temperature should contain virus with the H3N2 surface proteins but with a wild type gene derived from the HON1 parent replacing the gene with the ts lesion. Failure to derive wild type recombinants at 39°C would suggest that the original mutant contained a lesion on one of the surface glycorprotein genes. To date we have tested ts 315, 422, 463, 454 and 464 and have demonstrated that none of these viruses have ts lesions affecting their surface glycoproteins. In addition, the neuraminidase gene was shown not to be affected in the ts-2[C] recombinant.

2. Further transfer of ts lesions by recombination - In studies with the original ts recombinants our genetic analysis was complicated by the variable interactions between the ts-1[A], ts-1[E] and ts-1[H] viruses. In order to elucidate some of the factors which may have contributed to this varibility we undertook to make a second generation of recombinants between these viruses and either the Udorn/72 (H3N2) strain or the Marton/43 (HON1) strain. Analysis of the ts-1[E] recombinants produced in this manner indicated that the original H3N2 ts-1[E] virus contained 2 ts lesions--R1 and R8. Encouraged by this success, the ts-1[H] recombinant was recombined with the HON1 strain and ts mutants with the HO surface antigen were selected. These ts HON1 viruses were than analyzed genetically for the presence of the 3 lesions that the parent ts-1[H] virus was known to contain (group I; group II and group VII). Of the 20 recombinants thus far tested, only 2 appeared to have fewer lesions than the parental type. Both of these recombinants appeared to have lost the group II lesion. Studies on the reassortment of lesions in mutant ts-1[E] have also suggested that the group II lesion was less readily transferred by recombination than than the other lesion of this virus. Of interest, among the 9 HO-ts-1[H] recombinants tested there was a spectrum of shutoff temperatures from 37°C to 39°C. The parental H3N2-ts-1[H] had a shutoff temperature of 39°C.

Recent evaluation of the H3N2 ts-1[E] recombinants in doubly seronegative children revealed a low, but unacceptable, level of reversion to ts+ phenotype even though these viruses possess ts lesions on 2 segments of the influenza A virus genome. It was therefore necessary to identify additional ts mutants that fail to show such reversion. One possibility was the influenza A/HK/68-ts-1[A] virus which was shown to contain ts lesions in complementation groups 1 and 5 and to have an in vitro 37°C shutoff temperature. It was possible that the lower shutoff temperature and/or more genetically stable ts lesions might render this virus acceptably attenuated and genetically stable in children. In order to analyze the biological and genetic properties of HK/68-ts-1[A] virus it was mated with the Udorn/72 (H3N2) wild type virus and recombinants with the ts property of the ts parent and the hemagglutinin of the Udorn/72 virus were isolated. Genetic analysis of 8 recombinant clones identified three subsets of recombinant progeny; one subset possessed the same shutoff temperature as the ts parent (37°C) and contained 2 ts lesions, one in complementation group 1 and the other in group V; a second subset that also possessed a 37°C shutoff temperature but only the complementation group 1 lesion; and a third subset that had a 38°C-39°C shutoff temperature but only the complementation group V lesion. The clones of virus with the segregated single lesions were genetically stable after 5 passages in eggs. This is in contrast to the segregated single lesioned recombinant of the HK/68-ts-1[E] virus which was genetically unstable after passage in eggs. Biological evaluation revealed that these viruses were considerably restricted in replication in hamster lung, but grew well in the nasal turbinates. Clone 46, a double lesioned virus, has been the most extensively studied in the hamster lung and it failed to show evidence of genetic instability. Under the same conditions of evaluation the HK/68-ts-1[E] virus was unstable genetically. These results are sufficiently encouraging to warrant evaluation of the ts-1[A] recombinants in doubly seronegative volunteers to determine their attenuation, antigenicity and genetic stability.

3. Attenuation of wild type H3N2 influenza A virus by recombination with the Hong Kong/68-ts-1[E] temperature sensitive mutant - The production and analysis of Udorn/72-ts-1[E] recombinant clones was discussed in last years' report. We have examined further the two types of recombinant clones produced from mixed infection with A/Udorn/307/72 and Hong Kong/68-ts-1[E]. The first subset of recombinants was shown to possess the 2 ts lesions of the ts-1[E] parent, the same 38°C *in vitro* shutoff temperature of the parent, a similar level of restriction of growth in the hamster lung, and a similarly low but measurable frequency of reversion to the ts+ phenotype in the hamster lung. The second subset possessed only one of the ts lesions, had a 39°C *in vitro* shutoff temperature, was less restricted in growth in the hamster lung than the double lesioned ts-1[E] clones and exhibited a higher frequency of reversion than the double lesioned ts-1[E] clones. Recently, recombinant clones of Hong Kong/68-ts-1[E] and A/Georgia/101/74 (H3N2) were produced and analyzed. These recombinant clones exhibit behavior analogous to the Udorn/72-ts-1[E] clones consistent with the hypothesis that attenuation of new wild type strains can be achieved in a controlled manner by introduction of appropriate, defined ts lesions into new epidemic strains.

4. Production of a ts virus possessing two ts lesions by recombination of two genetically distinct ts donor viruses - We have shown that the Udorn/72-ts-1[E] recombinant clones that have lesions on two RNA segments exhibit more growth restriction and more genetic stability in hamsters and in volunteers than do clones with one lesion. Recombinants with 2 desirable lesions can now be made to order by combining mutations from 2 different ts influenza A mutants (Hong Kong/68-ts-304 and Hong Kong/68-ts-1 [E]) to produce a recombinant with ts lesions derived from the 2 ts donors. Initial studies with the new double mutant are encouraging with regard to growth restriction and genetic stability in the hamster lung.

5. Studies in the hamster with ts+ viruses produced from ts mutants - Ts+ viruses (possessing the temperature-sensitivity phenotype of wild type virus) were derived by two methods--reversion of ts virus in the hamster lung and recombination between two genetically distinct ts viruses. When ts+ revertants or ts+ recombinants were administered to hamsters, the following observations were made: 1) the ts lesion can be the sole determinant of growth restriction in the lung and by inference, attenuation; 2) some ts viruses may possess non-ts lesions that also restrict replication of virus in the lung; 3) ts lesions produce a greater restriction of growth

in the warmer lower respiratory tract (37°C) than in the cooler nasal turbinates while non-ts lesions do not produce such a selective restrictive effect. Finally, restriction of growth of ts viruses in the lower respiratory tract was attributable in most instances to the effect of the ts lesions.

6. Comparison of the genetic and biological properties of 5FU induced mutants and cold adapted influenza virus recombinants - Maassab, Davenport Kendal and their collaborators have taken an alternate approach to development of a live influenza virus vaccine. Rather than preparing ts mutants they have developed a cold adapted mutant and transferred the cold adapted lesion(s) into new antigenic variants. We have undertaken a comparison of the biological and genetic properties of the cold adapted parent (P17; derived from an H2N2-1960 parental strain) and 3 of its recombinants: CR6 (H3N2-Queensland/6/1972), CR12 (H3N2-A/AA/9/1973) and CR13 (H3N2-Dunedin/4/1972). We have confirmed that the process of cold adaptation of the H2N2-1960 parental virus resulted in the aquisition of the ts phenotype. The CR6 and CR12 recombinant viruses were also ts (their wild type parents grew equally well at the permissive and restrictive temperatures 32°C and 39°C, respectively). We were not able to prepare a pool of CR13 which showed reduction of replication at 39°C. Interestingly, the shutoff temperatures of the 3 ts viruses were different; P17, 37°C; CR6, 37°C; and CR12, 38°C. Preliminary study of the growth of these viruses in the hamster lung showed a relationship of shutoff temperature to level of replication. When compared with its homologous parent the reduction in growth in the lung was P17 10^{-5}; CR6 10^{-4} and CR12 10^{-3}. Maassab et al. had previously studied the biological behavior of the cold adpated variants in mice, ferrets and man and demonstrated that these viruses were attenuated when compared with the parental strains and that they had the ability to stimulate protective antibodies.

The genetic analysis of the 3 cold adapted ts viruses indicated that they all shared a ts lesion in the same complementation group (I). Thus these viruses shared a ts lesion with ts-1[A], ts-1[E] and ts-1[H]. All 3 cold adapted variants readily underwent genetic interaction with members of the other 6 complementation groups which we have described. We shall compare the genetic stability of the group I lesion derived from the cold adapted mutants with that derived from the 5FU derived H3N2 ts mutants. If the cold adapted mutant appears to be more stable we shall use it as a source of the group I lesion in preparing vaccine strains.

7. An immunological procedure to select recombinant progeny with the desired neuraminidase as well as the desired hemagglutinin - Immunity to the neuraminidase glycoprotein antigen has been shown to modify clinical illness resulting from infection with wild type and attenuated influenza A viruses. Recent studies suggest that anti-neuraminidase immunity can prevent infection as well as modify illness. Since attenuated vaccine anti-neuraminidase-antibody specific for the neuraminidase of the attenuated clones (i.e., ts parent) incorporated into the agar at an appropriate dilution suppressed growth of virus possessing the ts parental neuraminidase but allowed growth of virus with wild type neuraminidase. All plaques

picked in such a system [illegible] yield virus with neuraminidase of the wild type virus. This sytem was successful for crosses between viruses belonging to different subtypes, (i.e., N1 and N2) as well as viruses within one subtype ($N2_{68}$ and $N2_{74}$). Therefore, one can now readily select in a plaque system the recombinant progeny virus with the desired neuraminidase as well as the desired hemagglutinin.

(8) Protection from infection with a ts mutant by anti-neuraminidase immunity - It is generally accepted that anti-neuraminidase antibody modifies infection with an influenza A virus but does not prevent infection. Preliminary evidence suggests that the situation may be more complex. The 50% infectious dose (ID_{50}) of a ts mutant of influenza A virus was determined in hamsters with specific anti-neuraminidase immunity and in a control group. The ID_{50} was $10^{2.5}$ $TCID_{50}$ of virus for the control group and greater than 10^{4} $TCID_{50}$ for the hamsters with anti-neuraminidase immunity. Thus, hamsters with a level of neuraminidase antibody similar to that present in many members of our present population resisted infection with an attenuated ts mutant of influenza A virus.

9. Development and characterization of temperature sensitive mutants of influenza B virus - The rationale and approach to the development of ts mutants of influenza B virus were described in last year's annual report. Six ts mutants of influenza B/59 virus were isolated and characterized in terms of shutoff temperature, growth and genetic stability after replication in hamster lung and nasal turbinates. These mutants were shown to induce protection against wild type challenge. There was a definite correlation between level of replication, amount of antibody induced and protection afforded to wild type virus challenge.

The genetic analysis of ts mutants of influenza B ts mutants has proved more difficult than with influenza A mutants because wild type influenza B viruses (three distinct strains) are naturally temperature sensitive (i.e., they exhibit a 10-100 fold reduction in efficiency of plaque formation at the restrictive temperature of 39°C). Wild type influenza A viruses (three distinct strains) plaque with equal efficiency at permissive and restrictive temperature.

Our original plan was to isolate ts mutants of influenza B/59 virus and to transfer the ts defects to more current B strains, i.e., B/74 virus, that differ significantly in the surface hemagglutinin glycoprotein. This has been accomplished. Three B/59 mutants ts-78, ts-80 and ts-187 have been selected as donor viruses either because they were genetically stable after replication in hamsters or because they showed significant suppression of replication (i.e., greater than 100-fold more than wild type virus) in hamster pulmonary tissue. The ts defect(s) has been transferred to a wild type B/74 virus and 5 to 15 ts clones possessing the B/74 hemagglutinin have been isolated from each cross. These are being cloned before genetic and biological evaluation is undertaken. The experience to date indicates that ts lesions can be transferred with high efficiency from a B/59 ts virus to a B/74 wild type virus. This approach to live vaccine production for influenza B virus appears to be as feasible as it is for influenza A viruses.

Keyword Descriptors

Myxoviruses
Paramyxoviruses
Respiratory syncytial virus
Virus genetics
Attenuation

Publications

Hodes, D.S., Schnitzer, T.J., Kalica, A.R., Camargo, E., and Chanock, R.M.: Inhibition of respiratory syncytial, parainfluenza 3 and measles viruses by 2-deoxy-D-glucose. Virol. 63: 201-208, 1975.

Richman, D.D., Murphy, B.R., Tierney, E.L., and Chanock, R.M.: Specificity of the local secretory antibody to influenza A virus infection. J. Immunol. 113: 1654-1656, 1974.

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Murphy, B.R., Spring, S.B., Richman, D.D., Tierney, E.L., Kasel, J., and Chanock, R.M.: Temperature sensitive mutants of influenza virus. VII. Transfer of the ts-1[E] lesions to a wild type influenza A virus with HON1 surface antigens. Virology, in press.

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Richman, D.D., Murphy, B.R., Spring, S.B., Colemen, M., and Chanock, R.M.: Temperature sensitive mutants of influenza virus. IX. Genetic and biological characterization of ts-1[E] lesions when transferred to a 1972 (H3N2) influenza A virus. Virology, in press.

Serial No. Z01 AI 00025-14 LID
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual project report
July 1, 1974 through June 30, 1975

Project Title: Studies on *Mycoplasma pneumoniae*

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Total:	26/12		12/12	38/12
Professional:	14/12		12/12	26/12
Other:	12/12			12/12

Project Description

Objectives: (1) to study the infectivity, stability and protective capacity of ts mutants of *M. pneumoniae* (strain PI 1428) in the hamster animal model and in man; (2) to study the effect of route of administration, site of colonization, dose and particle size of inoculum on the development of *M. pneumoniae* disease and subsequent immunity; (3) to develop a simian model for *M. pneumoniae* disease for use in studies of pathogenesis and immunity as well as for vaccine safety testing.

Progress

(1) Natural History of Infection of Hamsters with ts Mutants

Hamsters were infected with wild type (ts+), ts-640, ts-H9 and ts-H10 mutants of *M. pneumoniae* and studied at intervals over a 519 day period. ts-640, ts-H9, and ts-H10 mutants produced an attenuated upper and lower respiratory tract infection in the hamster, (i.e., without evidence of pulmonary pathology), whereas ts+ *M. pneumoniae* organisms produced significant pulmonary disease. The ts mutants proved to be genetically stable *in vivo* over an 18 month period of infection. Infection with the ts+, ts-640, ts-H9, or ts-H10 organisms induced significant, prolonged resistance to subsequent challenge with virulent ts+ *M. pneumoniae*.

Animals infected with ts+ *M. pneumoniae* carried organisms in the upper and lower respiratory tract for a prolonged period following initial infection. Similarly *M. pneumoniae* ts mutants were detected in nasal turbinates and lungs of infected hamsters for at least 465 days. Such chronic carriage of *M. pneumoniae* was not associated with gross or histological evidence of pulmonary disease nor with untoward effects on the overall health of hamsters.

(2) Influence of Route of Administration of M. pneumoniae on Course of Experimental Infection

In collaboration with USAMRIID, Frederick, Md., hamsters were exposed to wild type *Mycoplasma pneumoniae* (MPn) aerosolized either as small particles (2.4 μm) for placement in the lower respiratory tract, or as large particles (10 μm) for upper airway deposition. The response of aerosol-exposed hamsters was compared to those infected by the conventional intranasal (IN) route to ascertain the effects of MPn presented to various regions of the respiratory tree. Hamsters infected IN with $10^{6.0}$ colony forming units (CFU) developed characteristic peri-bronchial pneumonia and moderate serum complementation-fixing (CF) antibody response; $10^{6.0}$ CFU of organisms per gm of lung were recovered 10-14 days post-infection. Animals exposed to a small particle aerosol (SPA) which delivered an inoculum of $10^{4.0}$ CFU developed equivalent titers of organisms in the lungs, but demonstrated less severe histopathology and failed to develop a significant serum CF antibody response. When a comparable dose ($10^{4.0}$ CFU) was administered IN hamsters developed a response similar to that seen in the SPA group indicating that the apparent attenuation associated with SPA was a dose related phenomenon. In contrast to the response seen following SPA, hamsters infected with large particle aerosol (LPA) ($10^{3.5}$ CFU) showed some colonization with MPn in the nasopharynx, but failed to develop infection in the lungs or a serum CF antibody response. When challenged IN with $10^{6.5}$ CFU of wild type organisms 40 to 50 days after initial exposure, the SPA and IN groups were both resistant; they exhibited minimal histopathology and very small amounts of MPn were recovered from the lungs. Relative to SPA and IN groups the LPA treated group showed less protection following IN challenge with virulent organisms.

In other studies oral administration of *M. pneumoniae* (ts+ and ts strains) has been shown to be an efficient and useful means of inducing an asymptomatic and protective infection in hamsters. The mechanism by which the oral route provides such protection is being explored.

(3) Experimental Infection of Rhesus Monkeys

Three seronegative young-adult rhesus monkeys were inoculated with *Mycoplasma pneumoniae* ($10^{7.4}$ CFU per animal) by oropharyngeal administation of coarseparticle aerosol. Five to six days after inoculation of the animals, specimens collected from the upper respiratory tract became positive for *M. pneumoniae*. Each animal subsequently developed serum antibodies as determined by complement-fixation, complement-mediated killing and tetrazolium-reduction inhibition techniques. Infection was subclinical and serial chest roentgenograms failed to disclose pneumonia throughout the course of infection. Blood counts and cold-agglutinin titers remained unchanged. Although *M. pneumoniae* was recovered from the upper respiratory tract of two monkeys for 50 days, there was no evidence of transmission of infection to cagemate controls or animal handlers. Attempts to infect other simians (squirrel monkeys) with *M. pneumoniae* are now underway.

Significance of Program to the Institute

M. pneumoniae is an important cause of lower respiratory tract disease, especially during the first 2 decades. Attempts to develop attenuated mutants for use in immunoprophylaxis and to achieve a better understanding of the pathogenesis of disease are relevant to the Institute's mission of disease prevention and control.

Proposed Course of Study

The effect of site of deposition and size of inoculum on the course of *M. pneumoniae* infection and disease will be studied more extensively next year. Information gained from such studies may prove helpful in the development of techniques for administration of live attenuated *M. pneumoniae* mutants for purposes of immunoprophylaxis. Use of a proper sized inoculum delivered to the appropriate region of the respiratory tract may prove decisive in achieving the desired goal of an asymptomatic, immunizing infection.

Keyword Descriptors

Mycoplasma pneumoniae
Pathogenesis
Immunology
Mycoplasmal genetics

Publications

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Serial No. Z01 AI 00026-08 LID
1. Infectious Diseases
2. Hepatitis Virus
3. Bethesda, Maryland

PHS - NIH
INDIVIDUAL PROJECT REPORT
July 1, 1974 through june 30, 1975

Project Title: Laboratory and Epidemiologic Studies of Viral Hepatitis Agents

Previous Serial No.: NIAID 70D

Principal Investigators: Dr. Robert H. Purcell
Dr. Jules L. Dienstag
Dr. John M. Peterson
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Other Investigators: Dr. Paul Holland, Dr. Harvey Alter (CC, Blood Bank, NIH)
Dr. John L. Gerin (MAN Laboratory)
Dr. William London (NINDS)
Dr. James Maynard (CDC)
Dr. Lewellys Barker, Dr. Douglas Lorenz (FDA)
Dr. Albert Kapikian (NIAID)
Dr. Marcel Conrad (Walter Reed)
Dr. Joseph Boggs (Children's Memorial Hosp., Chicago)
Dr. James Mosely (USC)
Dr. John Routenberg, Dr. Richard R. Hooper (Naval Recruit Center, San Diego)
Dr. Frederick Deinhardt, Dr. Jeffrey Cross (Pres. St. Lukes Hosp.)
Dr. Kenneth Soike (Albany Med. College)
Dr. William Allen (Delta Primate Center)
Dr. Thomas Edgington (Scripps Clinic and Res. Fdn.)
Dr. Robert G. Knodell (Walter Reed Hosp.)

Contract Support:

Contract Number	Project Off. NIAID	Contractor	Contractor's Proj. Dir.	Annual Funding Level
NO1 AI 12079	Dr. Purcell	Meloy Labs.	Dr. Verna	$ 212,704
Interagency Agreement	Dr. Byrne	Energy Res. & Develop. Admin.	Dr. J. Liverman	479,900
NO1 AI 12197	Dr. Purcell	Tulan Univ.	Dr. P. Gerone	$ 28,914
NO1 AI 32509	Dr. Tyeryar	Scripps Inst.	Dr. Edgington	$ 69,640

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	Total
Total:	84/12			84/12
Professional:	48/12	0/12	0/12	48/12
Other:	36/12		0/12	36/12

Project Description

Objectives: (1) To collect clinical material of high potential infectivity for use in laboratory studies: (2) To develop and test in vitro procedures and animal systems for use in laboratory studies of viral hepatitis; (3) To propagate the viruses of hepatitis in culture or in experimental animals; (4) To study the biochemistry, biophysics, immunology and epidemiology of hepatitis-associated antigens; (5) To develop vaccines or other control measures for viral hepatitis.

Methods Employed:

(1) Clinical Materials were collected from patients undergoing major heart surgery at NIH and from various animal and human populations through collaborative studies as described below.

(2) Hepatitis antigens were purified by density banding in a cesium chloride gradient, rate zonal centrifugation in sucrose and preparative electrophoresis.

Particles believed to be the virions of hepatitis A and B were isolated and analyzed.

(3) Samples containing hepatitis viruses were inoculated into various primate species and the animals were monitored for evidence of hepatitis for six months after inoculation.

(4) A wide variety of serologic techniques were used for seroepidemiologic studies.

Major Findings;

(1) Hepatitis A:

A. Serology - Despite significant progress in recent years in the study of hepatitis B, little progress was made in our understanding of hepatitis A because neither the virus nor infections caused by it could be identified. However, in a collaborative study with Dr. Albert Kapikian of this laboratory and Drs. Marcel Conrad and Dean Gibson of Walter Reed, we detected a virus-like particulate antigen in the stool of patients acutely ill with hepatitis A and demonstrated its temporal and serologic relationship to hepatitis A infection. The 27 nm particle is believed to be the hepatitis A virus. The antigen was detected, using the technique of immune electron microscopy, in stool samples collected only during the acute phase of infection. Using these particles as antigen, the development of antibody could be demonstrated during convalescence from hepatitis A infection.

More recently, Dr. Hilleman and his colleagues detected a similar virus-like particle in the serum and liver of marmosets experimentally infected with hepatitis, type A. Using antigen extracted from the livers of infected marmosets, they developed complement fixation and immune adherence tests specific for hepatitis type A virus infection. Although these tests, especially the immune adherence test, are important advances in the seroepidem-iologic study of hepatitis, type A, suitable marmosets for producing hepatitis A antigen are not available. For this reason we have attempted to adapt the immune adherence test for use with hepatitis A antigens derived from stools. We have purified hepatitis A antigens from acute phase stool samples obtained from infected Naval recruits involved in the San Diego hepatitis outbreak by a combination of ultracentrifugal and electrophoretic procedures. The resultant purified antigen was found to be immunoreactive in the immune adherence test and free of non-specific reactivity. In preliminary comparative tests, there was good agreement between serologic results obtained by immune electron microscopy and immune adherence.

B. Epidemiology - In collaboration with a number of other scientists we have tested sera obtained from hepatitis patients for serologic evidence of hepatitis A virus infection. Each of 42 individuals with epidemiologically compatible hepatitis type A developed a serologic response to hepatitis A antigen, as measured by immune electron microscopy. Included in this group of patients were individuals from 10 separate natural or experimental occurrences of hepatitis representing virtually all of the methods of exposure by which hepatitis A virus has been known to spread in the community: water-borne, food-borne, intrafamily, intrainstitutional and shell-fish associated. In contrast, only two of 103 patients with hepatitis not epidemiologically compatible with hepatitis, type A developed a serologic response. These two patients may have been infected through the use of illicit drugs. Included in the group of patients with non-A hepatitis were 30 with confirmed hepatitis, type B and 73 with non-B hepatitis.

In collaboration with Drs. Routenberg and Hooper, we have begun a serologic evaluation of the hepatitis A virus epidemic that occurred among naval recruits in San Diego, California. Several hundred clinical specimens, including stools, urines, saliva and semen samples, as well as sera, were collected during the course of the outbreak and transferred to the Laboratory of Infectious Diseases. Preliminary evaluation indicates that, of approximately 400 stool samples collected, many of which were serial samples from the same individuals, approximately 10% contain hepatitis A antigen as judged by immune electron microscopy. Some of the stools have very large numbers of particles present and are being used as a source of antigen for purification and subsequent serologic and biophysical studies. Each of 25 recruits with hepatitis studied developed antibody to hepatitis A antigen (anti-HA) in their convalescent sera. Selected clinical specimens are currently being prepared for evaluation of their infectivity in seronegative chimpanzees. These pools will serve as standard reagents of known infectivity titer for use in subsequent collaborative studies.

Using immune electron microscopy for the detection of anti-HA, we have begun an evaluation of age-specific patterns of anti-HA. Fifty nine percent of 83 serum samples from individuals of all ages and from various parts of the United States contained anti-HA; there was a direct relationship between age and frequency of such antibody. Thus, anti-HA was uncommon in children and young adults, a finding in agreement with similar preliminary serologic results obtained by Dr. Hilleman's group, using immune adherence assays. These findings suggest that the epidemiology of hepatitis, type A has changed in recent decades, probably as a result of improved public health measures. In collaborative studies with Drs. Boggs and Conrad, we have retrospectively examined the temporal relationship of HA Ag particle shedding to the development of hepatitis, type A in volunteers experimentally infected with hepatitis A virus at the Joliet Prison. HA Ag was first detected in the stool several days before transaminase values became elevated and could no longer be detected by the time peak transaminase activity was reached. Thus, this study demonstrated the need for early collection of stool specimens for the demonstration of fecal HA Ag.

In a series of collaborative studies with Drs. Barker, Lorenz, Maynard, Deinhardt and Cross, we have confirmed the lack of serologic relatedness between hepatitis A virus and the GB agent of Deinhardt and a lack of serologic relatedness between hepatitis A antigen and the fecal antigen of Cross. Additional studies are being carried out on the GB agent in attempts to determine whether it is a marmoset virus or a virus of human origin. These studies include inoculation of marmosets and chimpanzees with GB agent and attempts to develop a serologic test for identifying GB virus infection.

C. Biophysical Characterization - The hepatitis A antigen was reported previously by us to have a buoyant density in cesium chloride of approximately 1.4 gm/cc and a sedimentation coefficient in sucrose consistent with its size of 27 nm. In subsequent studies, Dr. Maynard and his colleagues reported the finding of multiple peaks of HA Ag at densities ranging from 1.32 to 1.40 gm/cc in cesium chloride. We have subsequently confirmed this finding of multiple densities for HA Ag and are purifying each density independently for serologic comparison.

In collaborative studies with scientists of the Bureau of Biologics and the CDC, livers from marmosets experimentally infected with hepatitis, type A were homogenized and examined for hepatitis A antigen. HA Ag with an isopycnic density of 1.34 gm/cc was detected in 3 of 4 marmoset livers, thus confirming the findings of Dr. Hilleman's group. In attempts to find a substitute for marmosets for such studies, seronegative chimpanzees experimentally infected with hepatitis, type A were subjected to subtotal hepatectomy and their livers studied for the presence of HA Ag. Hepatitis A antigen was detected in each of 2 livers studied, but it was present in concentrations too low to be useful for immune adherence studies. Bile collected during one such total hepatectomy was found to contain HA Ag with a density of 1.4. Thus, HA Ag has been detected in the liver of experimentally infected marmosets and chimpanzees, in the bile of experimental chimpanzees, and in the stool of experimentally infected chimpanzees and experimentally or naturally infected humans. The significance of the different densities of HA Ag observed in these studies remains to be determined, but they may represent complete and incomplete forms of the virion. Such multiple densities have also been described for parvoviruses, a family of viruses that HA Ag closely resembles.

D. Transmission studies in non-human primates - Hepatitis A virus has been successfully transmitted to marmosets and chimpanzees; infection results in biochemical and histopathologic evidence of hepatitis in both species. Since both chimpanzees and marmosets are expensive and difficult, if not impossible, to obtain, we, in collaboration with Dr. London, are attempting to transmit hepatitis A virus to other species of non-human primates. Serum samples from 6 species of primates (rhesus monkeys, patas monkeys, African green monkeys, baboons, cebus monkeys, owl monkeys) have been tested for anti-HA by immune electron microscopy and/or immune adherence. One or more animals of the following species were found to have such antibody: rhesus monkey, owl monkey, patas monkey, African green monkey. Seronegative representatives of each of these species tested, plus other species as they become available for testing, are being inoculated with hepatitis A virus and being monitored for serologic, biochemical and histopathologic evidence of infection. Rhesus monkeys developed serologic evidence of infection in a preliminary study, but we have been unable to confirm this in a subsequent study. Results of studies on other non-human primates are not yet available.

The chimpanzee appears to be an excellent model for the study of hepatitis, type A. The temporal relationships of HA Ag shedding in the stool, development of histopathologic lesions in the liver, serum transminase elevations and development of anti-HA have been studied, using immune electron microscopy assays. HA Ag particles were shed in the stool during the late prodrome and early acute phase of infection and they were no longer detectable by the time transaminase activity reached maximum levels. Similarly, anti-HA was first detected during the early acute phase, concident with the beginning of liver damage, and the level of such antibody rose slowly over the succeeding months. The early development of anti-HA suggests that immunopathologic factors may be important in the clinical expression of hepatitis, type A. The results of these studies were remarkably similar to those obtained in the study of experimental hepatitis A virus infection in Joliet prisoners.

E. Reference reagents. Experimentally infected chimpanzees were plasmapheresed prior to infection and during convalescence. Serum obtained from these plasmapheresis units were pooled, serologically evaluated and packaged by the Research Resources Branch of NIAID for distribution to the scientific community. Final evaluation of the packaged reagents is currently in progress. Preliminary evaluation indicates that the preinfection serum is free of anti-HA and the convalescent serum has an anti-HA titer of 1:500-1:1000 by immune electron microscopy and 1:640 by immune adherence. These reagents will be used on an interim basis until hyperimmune serum can be prepared against HA Ag.

F. Tissue culture - Efforts to isolate hepatitis A virus in tissue and organ culture will be increased. Attempts to determine which of the densities of HA Ag is associated with infectivity are currently in progress; virus-like particles of the appropriate density will be purified to remove possible interfering particles and tested for infectivity in tissue culture and seronegative chimpanzees

2. Hepatitis B.

A. Serology - A radioimmunoprecipitation test for antibody to hepatitis B core antigen (HB_c Ag) was developed. Cores of Dane particles were purified from human plasma and intrinsically labeled with tritiated thymidine via the DNA polymerase associated with such cores. The radioimmunoprecipitation test was found to be 300-1000 times more sensitive than complement fixation for measuring antibody to cores (anti-HB_c). Studies of the dynamics of antibody formation following hepatitis B virus infection are currently in progress. In the course of these studies, it was found that two populations of Dane particle cores, a heavy population with a buoyant density in cesium chloride of approximately 1.36 gm/cc and a light population with a density of approximately 1.32 gm/cc exists. The specific activity of DNA polymerase is much higher in the heavy cores than in the light cores. It is probable that the heavy cores were extracted from infectious Dane particles, whereas the light cores were probably extracted from incomplete viral particles.

B. Epidemiology - In collaboration with Drs. Holland and Alter of the Clinical Center Blood Bank, we have continued studies of post-transfusion hepatitis occurring among patients undergoing corrective cardiac surgery at NIH. We have confirmed the much greater risk of receiving blood derived from commercial sources when compared with voluntarily donated blood. The frequency of hepatitis type B has been markedly reduced by the use of only volunteer blood, coupled with screening of all blood for HB_s Ag by radioimmunoassay procedures. Nevertheless, hepatitis, type B has been documented in individuals who received only screened volunteer blood. Attempts to find ways of detecting HB_s Ag negative, but still infectious, donors are in progress.

C. Transmission to non-human primates - (1) Rhesus monkeys. We have been unable, during the past year, to expand our studies of hepatitis B virus infection in rhesus monkeys because of an inability to demonstrate successful transmission in recent experiments. The reason for this problem is unclear, but may be related to loss of infectivity of existing pools of virus and/or diminished susceptibility of rhesus monkeys available for testing. Attempts to resolve this problem are in progress.
(2) Transmission to chimpanzees. In collaborative studies with Drs. Barker, Maynard and London, infectivity titration of pools of hepatitis B virus was continued. Pools of subtype ayw and adw were both found to have infectivity titers of approximately $10^{7.5}$ chimpanzee infectious dose_{50} units per ml. A pool of HBV of subtype adr has an infectivity titer of greater than 10^6, and a pool HBV, subtype ayr has a titer of

greater than 10^1 but less than 10^4. Additional titration studies of these latter two pools are still in progress. Preliminary results of cross-challenge experiments with these pools in chimpanzees indicates infection with one subtype of HBV confers partial, if not complete, protection against rechallenge with another subtype.

D. Immunoprophylaxis -
(1) Passive immunization. In collaborative studies with Drs. Barker, Maynard and London, the protective effect of high titered hepatitis B immune serum globulin is being evaluated under controlled conditions in chimpanzees inoculated with known doses of HBV. Preliminary results indicate little, if any, protective effect of hepatitis B immune serum globulin against HBV infection or illness, but additional studies with more chimpanzees must be carried out.
(2) Active immunization. Based upon results of studies of hepatitis B virus in chimpanzees and rhesus monkeys and results of biophysical and immunological studies of hepatitis B antigen, an experimental hepatitis B vaccine was prepared from purified hepatitis B antigen obtained from human chronic carriers of the antigen, in a collaborative study with Dr. Gerin). Immunologic stability of the antigen following exposure to several different potential methods of inactivation was studied, using immunogenicity extinction tests in guinea pigs. Immunogenicity was retained by the antigen following all inactivation methods. Inactivation with formalin was chosen, and safety tests of inactivated and uninactivated vaccine preparations were carried out in seronegative chimpanzees in collaboration with Drs. London, Soike and Allen. None of the vaccinated chimpanzees developed evidence of hepatitis B virus infection, indicating that the purification procedure removed and/or inactivated hepatitis B virus.

Twenty-four weeks after vaccination, vaccinated and control chimpanzees were challenged with live hepatitis B virus. Each control chimpanzees developed hepatitis, type B. In contrast, three chimpanzees vaccinated with an *ayw* subtype vaccine were completely resistant to infection with *ayw* virus, and one chimpanzee vaccinated with an *adr* subtype vaccine was protected from hepatitis but did experience an inapparent HBV infection characterized by development of anti-HB_c and anti-HB_s. Thus, hepatitis B vaccine appeared to be safe and effective against hepatitis, type B.

Based upon these encouraging results, larger batches of hepatitis B vaccine, of subtypes *adw* and *ayw*, are being prepared in collaboration with Dr. Gerin for subsequent expanded safety and efficacy tests in chimpanzees, to be followed by testing in humans.

E. Tissue culture - A renewed effort is being made to isolate hepatitis B virus in cell culture. New knowledge about the structure of the hepatitis B virion is being applied to the purification of heavy (presumably fully infectious) Dane particles devoid of light (presumably defective) particles and free surface antigen. Pools of such materials will be tested for infectivity in seronegative chimpanzees and employed as inocula for isolation studies. Evidence of viral replication will be sought, using the many serological and biochemical tests now available.

(3) Non-A, NonB Hepatitis

We have documented the existence of post-transfusion hepatitis not serologically related to hepatitis types A or B or CMV or EBV infection. Such non-A, non-B hepatitis was found to occur, like hepatitis, type B, approximately 10 times more frequently following transfusion of commercial blood as following transfusion of voluntarily donated blood. Epidemiologically, non-A, non-B hepatitis resembles hepatitis, type B more closely than it does hepatitis type A: transmission to contacts is rare, and a proportion of cases progress to chronic hepatitis. Approximately three-quarters of icteric and anicteric hepatitis occurring in patients following corrective cardiac surgery is non-A, non-B hepatitis at present.

A. Serology - Attempts to detect an antigen-antibody system specific for non-A, non-B hepatitis are in progress. These include attempts to develop a solid-phase radioimmunoassay, using acute phase sera from non-A, non-B hepatitis patients as a potential source of antigen and convalescent sera from the same patients as a source of antibody. IgG was purified from convalescent sera from such patients and radiolabeled with ^{125}I. Radioimmunoassays employing acute and convalescent serum samples from 11 non-A, non-B hepatitis patients have been tested for reactivity; to date no significant reactivity has been detected. Since non-A, non-B hepatitis patients may not mount a vigorous primary immune response (analogous to the poor anti-HB_s response of hepatitis B patients) we have purified IgG from hemophiliacs and other multiply transfused individuals who might have had multiple exposures to non-A, non-B hepatitis. These IgG reagents are being employed as radioactive probes in a search for non-A, non-B hepatitis antigens in acute phase sera from non-A, non-B hepatitis patients.

Similarly, acute and convalescent sera from patients with non-A, non-B hepatitis are being tested in immune electron microscopy tests in a search for virus-like particles. To date none have been found.

B. Epidemiology - A significant proportion of hepatitis previously thought to be hepatitis, type A has been shown to be, in reality, hepatitis, type B. Thus, up to 40% of "sporadic" hepatitis has been found to be HB_s Ag positive in some studies. The remaining 60% of "sporadic" hepatitis does not resemble hepatitis, type A epidemiologically. In collaborative studies with Dr. Mosley, we have shown that most of this "sporadic" hepatitis is not serologically related to hepatitis, type A, CMV or EBV. Thus, it appears to be non-A, non-B hepatitis.

C. Transmission to Non-human Primates - In collaborative studies with Drs. Alter and London serum samples from patients acutely or chronically ill with non-A, non-B hepatitis have been inoculated into chimpanzees in an attempt to transmit an etiologic agent. The animals did not demonstrate histopathologic or biochemical evidence of hepatitis. However, we cannot rule out an inapparent infection, since serologic methods of diagnosis are not available.

Keyword Descriptors

Hepatitis, type B, Hepatitis, Type A
Vaccine
Hepatitis B virus
Hepatitis A virus
Radioimmunoassay
Immune adherence assay
Ultracentrifugation
Serology
Epidemiology
Non-human primates

Publications

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Serial No. Z01 AI 00027-8 LID
1. Infectious Diseases
2. Mycoplasma Section
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Basic Studies on Mycoplasmas

Previous Serial No.: NIAID 70F

Principal Investigator: Dr. Joseph G. Tully

Other Investigators: None

Cooperating Units: Bureau of Biologics, FDA, Bethesda, Maryland
(Dr. M.F. Barile)
Wistar Institute, Philadelphia, Pennsylvania
(Dr. H. Fred Clark)
Dr. R.M. Cole (Serial No. Z01 AI 00004-15 LSD)

Man Years:	NIAID Lab. Staff.	Other NIH Personnel	Guest Workers	Total
Total	36/12	-	-	36/12
Professional	12/12	-	-	12/12
Other	24/12	-	-	24/12

Project Description

Our objectives cover both basic and applied aspects of mycoplasmas and related wall-free procaroytes, including their toxins and other biological factors involved in virulence, their immunological inter-relationships, and their possible role in human infection or in disease of uncertain etiology.

We have continued and extended the study on the purification and chemical characterization of neurotoxin from Mycoplasma neurolyticum. Variability in toxin levels of crude toxin prepared from the toxigenic strain (type A) led to more detailed examination of the relationship of growth phase of the organism to elaboration of the so-called exotoxin. Potency of whole cell broth culture toxin was directly associated with the log phase of growth and release of cell-free toxin did not appear to be associated with cold shock or lysis of cells. Centrifugation and/or ultrafiltration through 100 nm membrane filters of whole cell broth cultures revealed differences in amounts of toxin and the manipulations themselves seemed to alter potency. There would now seem to be serious questions about whether the toxin is truly an exotoxin. Recent tests have also indicated that significant amounts of toxin are adsorbed to membrane filters during ultrafiltration preparative techniques. Tests with parabiotic chambers, separated by 100 nm membranes, should give some indication whether elaboration of toxin occurs into a cell-free environment in the absence of physical forces capable of releasing toxin from cells.

The ultrastructure of toxigenic and non-toxigenic strains of M. neurolyticum was also examined. Negatively stained and sectioned cells of representative cultures indicated that the toxic strain consisted of rather uniform spherical cells (some devoid of cytoplasm), while the non-toxic strain comprised variable and often filamentous, dense cells. These studies are continuing in the hope that we might be able to identify toxin-containing cells by appropriate immuno-electron microscopic techniques or determine how toxin is released from cells. (Project No. Z01 0000415 LSD, Cole)

Column chromatography of crude toxin has successfully separated toxin from most medium protein but some loss in potency has been observed. Efforts are now being directed to other gel filtration techniques to see whether loss in specific activity of toxin can be reversed and improved yield of highly potent material made available.

The neurotoxicity of crude toxin from the Type A strain and comparable materials from a non-toxigenic strain (PG-28) have been performed in monkeys. The results showed that Type A toxin produced central nervous system (CNS) responses (ataxia, flaccid paralysis of limbs, and death from an ascending paralysis) with dosages of 1.5-1.7 gms/kg. No comparable CNS response was seen in monkeys receiving the non-toxic strain, although a shock-like syndrome and death was noted in monkeys receiving the upper range of the challenge dose. This latter response is probably associated with high salt or protein content of the crude materials. More purified toxin should provide clear confirmation of the role of toxin in these CNS responses. (Bureau of Biologics - Dr. M.F. Barile)

Studies on the suckling mouse cataract agent (SMCA) were carried out this year in collaboration with Dr. Fred Clark, Wistar Institute. The agent, previously considered to be a slow virus, was found to have the morphology and ultrastructure of mycoplasmas. Although the agent has not been grown in culture, it offers a useful model to study mycoplasma pathogenicity. Initial recovery of the agent was from a pool of rabbit ticks and our interest centered around the possibility that the agent might be a pathogenic form of spiroplasma. Four antisera prepared to chick embryo grown SMCA were examined in growth inhibition serological tests against Spiroplasma citri. Three sera had low levels of antibody to S. citri (2 mm inhibition) while one antiserum possessed moderate levels (4 mm) of antibody. Pre-immunization sera of each rabbit showed no activity. Adsorption of selected sera with dried S. citri removed the growth-inhibition activity of each serum. Further tests with the adsorbed sera are being carried out using neutralization tests against SMCA to confirm the specificity of the S. citri adsorption. Culture attempts with newly-defined medium for spiroplasmas may provide necessary factors to isolate and characterize the agent.

Keyword Descriptors

Mycoplasmas
Neurotoxin
Spiroplasmas
Mycoplasma structure and function

Publications

Tully, J.G., Barile, M.F., Edward, D.G. ff., Theordore, T.S., and Ernø, H.: Characterization of some caprine mycoplasmas, with proposals for new species, Mycoplasma capricolum and Mycoplasma putrefaciens. J. Gen. Microbiol. 85; 102-120, 1974.

Tully, J.G., Barile, M.F., Cottew, G.S., Edward, D.G. ff., Freundt, E.A., Gourlay, R.N., Jordan, F.T.W., Lindley, E.P., Razin, S., Whittlestone, P., and Bogel, K.: The FAO/WHO Programme on Comperative Mycoplasmatology. Veterinary Record 95: 457-461, 1974.

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1. Infectious Diseases
2. Respiratory Viruses &
   Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Study of Respiratory Viruses and Mycoplasmas in Volunteers

Previous Serial No.: NIAID 72B

Principal Investigators: Dr. Brian R. Murphy
Dr. Douglas D. Richman
Dr. Charles M. Helms
Dr. Michael B. Grizzard
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Cooperating Units: Dr. Louis Potash, Flow Laboratories
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Dr. Robert H. Parrott and Dr. H.W. Kim, Children's Hospital of D.C.
Dr. Gordon Douglas, Univ. Rochester
Dr. Neil Blacklow, Boston University

Contract Support:

Contrat Number	Project Off. NIAID	Contractor	Contractor's Proj. Dir.	Annual Funding Level
N01 AI 32521	Dr. Chanock Ms. Mattheis	Flow Labs.	Dr. Gilden	$ 490,000
N01 AI 32503	Dr. Chanock	Univ. Md.	Dr. Hornick	516,094
N01 AI 12091	Dr. Chanock	Res. Fdn. Ch. Hosp. D.C.	Dr. Parrott	198,336
N01 AI 22503	Dr. Murphy	Univ. Rochester	Dr. Douglas	167,232
N01 AI 32505	Dr. Chanock	Univ. Hosp. Boston	Dr. Blacklow	64,284

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	Total
Total:	60/12			60/12
Professional:	24/12			24/12
Other:	36/12			36/12

Project Description

Objectives: (1) To evaluate temperature sensitive mutants and recombinants of influenza A virus for evidence of attenuation in volunteers; (2) to characterize the serum and local respiratory tract anti-neuraminidase antibody response of man to infection with wild type or attenuated influenza A virus; (3) to evaluate the infectivity and clinical response to ts mutants of *M. pneumoniae*.

Major Findings

(A) Evaluation of ts Recombinants Derived from Hong Kong/68-ts-1[E] and Wild Type Strains of the H3N2 Influenza A Subtype

Studies were completed last year with ts clones produced by recombination of wild type A/Udorn/307/72 virus and Hong Kong/68-ts-1[E] (which has been shown to possess two ts lesions). Two subsets of virus possessing the Udorn/ 72 hemagglutinin and the ts phenotype were characterized. The first subset, of which clones 16 and 24 were the prototypes, possessed both ts lesions (complementation group I and II) and the 38°C shutoff temperature of the Hong Kong/68-ts-1[E] parent. Evaluation in hamsters revealed that the parent Hong Kong/68-ts-1[E] virus and the genetically equivalent recombinant Udorn clones 16 and 24 were similar to each other in the following respects: (1) level of replication in lungs and nasal turbinates; (2) low level of reversion to ts+ phenotype in the lungs; and (3) protection induced to homologous wild type virus challenge. The second subset, of which clone 13 was the prototype, possessed only one of the two ts lesions present in the Hong Kong/68-ts-1[E] donor and had a 39°C shutoff temperature. In hamsters, clone 13 was less stable genetically and was less restricted in replication in the lung than clones 16 and 24 which possessed both ts lesions of ts-1[E] virus. Volunteer studies also demonstrated that the single and double lesioned Udorn/72 recombinants exhibited a difference in virulence. The Udorn/72 clone 13 (39°C) virus retained the capacity to cause mild febrile influenzal illness, as had been shown previously with 39°C shutoff Hong Kong/68 mutants, but was attenuated in comparison to wild type influenza virus. This level of attenuation could not be explained as an effect of tissue culture passage of the clone 13 virus since in an additional study, 10^4 $TCID_{50}$ of wild type Udorn/72 virus at the same passage level as the clone 13 virus produced febrile influenzal disease which was considerably more severe than the mild influenzal illness caused by 10^7 $TCID_{50}$ of the clone 13 ts recombinant.

Two clones of Udorn/72-ts-1[E] (clone 16 and 24) which contained both ts-1[E] lesions (i.e., group I and group II) were administered to seronegative adult volunteers and were found to be as attenuated and antigenic as the Hong Kong/68-ts-1[E] donor virus. They were satisfactorily attenuated, stable genetically, antigenic, and they provided protection against a wild type virus challenge. These results provide further support for the concept that the ts lesions confer attenuation, and that viruses that have a specified ts lesion or set of lesions will behave predictably in man. This view gained further support from a limited trial in man of a recent ts-1[E] recombinant. The two ts lesions of ts-1[E] were transferred from the Hong Kong/68-ts-1[E] virus to a wild type influenza A/Georgia/101/1974 virus. This recombinant ts virus was suitably attenuated and antigenic when given to 4 seronegative volunteers. In contrast, the wild type

Georgia/74 virus induced febrile influenzal disease. In summary, the 2 ts-1[E] lesions have been transferred 3 times to a wild type virus bearing a new hemagglutinin and in each instance the new ts recombinant exhibited a similar level of attenuation and antigenicity for seronegative adults. Thus, it seems likely that transfer of the ts-1[E] lesions to any new influenza A virus will predictably result in attenuation of the recombinant possessing the new surface antigens.

(B) Evaluation of the Influenza A/HK/68-ts-1[E] Virus in Children
Extensive study of this virus in adults revealed that it had the following properties desirable for a live virus vaccine: (1) satisfactory infectivity for seronegative adults; (2) satisfactory attenuation for adults; (3) capacity to stimulate local and serum anti-hemagglutinin (HI) and anti-neuraminidase antibodies (ANAB) in seronegative volunteers, (4) stimulation of resistance to virulent, wild type virus; (5) relative genetic stability *in vivo*; (6) lack of communicability, (7) replication to high titer in avian leucosis virus-free eggs; (8) failure to induce alterations in pulmonary function, (9) safety and antigenicity in the elderly and chronically ill; and (10) localization of the ts lesions to genes that do not code for the hemagglutinin and neuraminidase. Because of need to develop effective immunoprophylaxis against influenza A virus in children, this virus was subsequently administered to this age group. Each of the adult vaccinees studied previously lacked serum HI antibody but possessed a measurable level of serum ANAB. Accordingly, we were particularly interested and concerned about the response of young children who might lack serum ANAB as well as serum HI antibody. Since neuraminidase immunity is effective in reducing the clinical response to infection with wild type influenza virus, it might also do so for the attenuated ts recombinant viruses. Indeed the full expression of virulence of the ts recombinant or any live influenza virus vaccine may be manifest only in HI antibody and ANAB negative individuals, i.e., doubly seronegative persons. Further, it would be important to determine the immune response of doubly seronegative young children to viruses containing the ts-1[E] genetic lesions because of the potential usefulness of such recombinants for vaccination in this age group and as a model for the antigenicity of an attenuated vaccine directed against the next pandemic variant which might possess new glycoprotein hemagglutinin and neuramindase surface antigens.

The Hong Kong/68-ts-1[E] was administered intranasally at the Children's Hospital of D.C. to 21 children at a dose of 10^5 TCID$_{50}$ by Dr. Kim and Dr. Parrott. One group contained 15 children (5-11 years of age) who lacked serum antibody to the hemagglutinin (<1:8), but who possessed serum antibody to the neuraminidase antigen. The second group included 6 children (2-4/12 to 3-10/12 years of age) who lacked serum antibody to both hemagglutinin and neuraminidase surface antigens of the influenza A virus, i.e., doubly seronegative children. Twelve of the 15 children in the first group were infected, but only one child developed mild rhinitis, 6 of the 12 infected vaccinees shed virus for a short interval, while 11 of the group developed an immunologic response. In contrast, each of the

6 vaccinees who lacked serum antibody for both surface antigens of virus shed a relatively larger quantity of virus over a longer interval than the first group; in addition, 4 children developed a transient febrile response during infection and 3 had mild rhinitis. Two children who lacked serum antibody to both surface antigens of influenza virus were given the influenza A/Udorn/72-ts-1[E] recombinant virus, which had the same genetic lesions as the Hong Kong/68-ts-1[E] recombinant virus. Both of the children were infected and one developed a transient fever. None of the 23 vaccinees given the Hong Kong/68-ts-1[E] or Udorn/72-ts-1[E] recombinant at children's Hospital of D.C. developed signs or symptoms of lower respiratory tract involvement. An additional 18 doubly seronegative children were given 10^4 $TCID_{50}$ of the Hong Kong/68-ts-1[E] virus at Vanderbilt University and the reactogenicity of the virus was reduced although the virus maintained its antigenicity.

These findings suggest that anti-neuramindase immunity naturally acquired from previous infection provided some protection against illness produced by the candidate vaccine virus. Only when doubly seronegative children were evaluated did it become apparent that the ts-1[E] recombinant viruses retained some virulence. Some of the children developed a transient febrile response not associated with any signs of lower respiratory tract involvment. It seems likely that a complete assessment of the virulence of candidate live influenza A vaccine viruses can only be made in individuals who lack immunity to both surface anrigens of the influenza A virus. It is important to achieve attenuation for such persons especially if one plans to use live attenuated vaccines to control pandemic disease caused by new antigenic variants of influenza A virus. If the next pandemic shift involves the hemagglutinin and neuraminidase antigens, every member of the population might resemble the doubly seronegative child in lacking immunity to both surface antigens. In this circumstance viruses which were acceptably attenuated for individuals with neuraminidase immunity might produce febrile responses similar to those induced by the ts-1[E] recombinants in doubly seronegative children.

The ts-1[E] recombinants were shown recently to contain discrete ts lesions which were located on 2 different RNA pieces of the segmented genome. Despite the existence of ts lesions of 2 separate RNA segments of the viral genome, the recombinants exhibited evidence of genetic instability during infection of 8 doubly seronegative young children during the studies at Children's Hospital of D.C. These revertants, which could not be detected in the original nasopharyngeal swab specimen, represented only a very small proportion of the virus population (10^{-2} to 10^{-3}) produced during infection of doubly seronegative children and the emergence of revertant virus under these conditions was not associated with lower respiratory tract disease. However, four of the 18 children who received 10^4 $TCID_{50}$ of HK/ts-1[E] virus during the studies at Vanderbilt University shed revertant virus which was detectable by assay of their original nasopharyngeal wash specimens. In contrast, the ts-1[E] recombinants were stable genetically when isolated from a considerably larger group of 78 adults and 15 older children who lacked serum HI antibody but who possessed neuraminidase antibody. It appeared that emergence of ts+ revertants occurred only in the absence of immunity to both hemagglutinin and neuraminidase antigens; presumably only in this circumstance did vaccine virus infection occur without restriction.

In view of the occurrence of reversion and low-grade fever, studies with the ts-1[E] recombinants in children have been suspended. Although the ts-1[E] recombinants did not prove to be the ideal donor of ts lesions for a live influenza vaccine they did prove helpful in establishing new standards to be required for future ts influenza vaccine viruses -- namely genetic stability in doubly seronegative individuals and failure to induce a febrile response in such persons. Other ts mutants which contain 2 independent ts lesions in regions of the genome which do not code for the hemagglutinin or neuraminidase antigen are currently under study and several appear to be more stable genetically than the ts-1[E] virus. These more stable recombinants will be evaluated in volunteers shortly.

(C) Evaluation of ts-H43 Mutant of *M. pneumoniae*

As reported last year the ts-H43 mutant, which has a shutoff temperature of 37°C *in vitro* was tested initially in 15 men who lacked serum MI antibody. They were inoculated intransally with 10^7 CFU of ts H43 by DeVilbis atomizer. During the 28 day period of intense medical surveillance none of the men developed signs or symptoms of illness. Each of the 15 volunteers developed evidence of infection and all of the isolates recovered from the men retained the ts phenotype. Radioimmunoprecipitation analysis of the nasal wash specimens indicated that 53% of the volunteers developed a local antibody response in the nose to ts-H43. 11 of the 13 men (94%) who could produce sputum-saliva specimens demonstrated a significant local secretory IgA response.

Encouraged by these findings, an additional 39 volunteers were given the ts-H43 candidate vaccine strain this year. Thus, a total of 35 seronegative and 19 seropositive volunteers have received 10^6-10^7 CFU of the ts-H43 mutant by the intranasal route. Ninety seven percent of the seronegative volunteers and 79% of the volunteers with a pre-existing MI antibody titer of 1:2 or greater were infected. Each of the isolates recovered from the volunteers retained the ts phenotype. Although febrile respiratory tract disease did not occur, bronchitis without fever developed in 4 of the 54 volunteers. Studies to determine the minimal infective dose are now in progress using inocula containing lower concentrations of the organism. Volunteers given 10^4 or 10^5 CFU of ts-H43 did not develop any signs or symptoms of illness.

Of interest was the observation made in collaboration with Fernald that none of the 49 men infected by 10^6 - 10^7 CFU of the ts-H43 mutant developed systemic cell mediated immunity to *M. pneumoniae*. In contrast, almost all of the volunteers developed a serum antibody response and almost all of those tested developed a respiratory tract secretory antibody response. We and others have suggested that M. pneumoniae disease results in part from an immunologic response to infection by individuals who were sensitized to the organism during prior infection. Fernald and Clyde have suggested that cell mediated immunity may play a role in the immunopathology of this disease. If this is so then the failure of ts-H43 to stimulate this type of response may offer a significant advantage to its use for immunoprophylaxis of M. pneumoniae disease.

Keyword Descriptors

Respiratory viruses
Respiratory mycoplasmas
Experimental infection in volunteers

Publications

Greenberg, H., Helms, C.M., Brunner, H., and Chanock, R.M.: Asymptomatic infection of adult volunteers with a temperature sensitive mutants of Mycoplasma pneumoniae. Proc. Nat. Acad. Sci. 71: 4015-4019, 1974.

Murphy, B.R., Hodes, D.S., Nusinoff, S.R., Stewart, S., Tierney, E.L., and Chanock, R.M.: Temperature sensitive mutants of influenza virus. V. Evaluation in man of an additional ts recombinant virus with a 39°C shutoff temperature. J. Infect. Dis. 130: 144-149, 1974.

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1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1974 - June 30, 1975

Project Title: Studies on ts Mutants of Streptococcus pneumoniae

Previous serial No.: None

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Dr. Michael B. Grizzard
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Other Investigators: Dr. Benjamin Prescott (LMI, NIAID)
Dr. Allen Cheever (LPD, NIAID)
Dr. Gerald Schiffman (Downstate Medical Center, S.U.N.Y.)

Cooperating Units: Laboratory of Microbial Immunity and Laboratory of Parasitic Diseases, NIAID
Downstate Medical Center, State University of New York

Contract Support:

Project Number	Project Officer NIAID	Contractor	Contractor's Proj. Dir.	Annual Funding Level
N01 AI 42541	J.C. Hill	S.U.N.Y.	G. Schiffman	$ 83,333

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	Total
Total	26/12			26/12
Professional	14/12			14/12
Other	12/12			12/12

Project Description

Objectives: S. pneumoniae, the primary bacterial pathogen of otitis media, continues to be a major cause of hearing loss in early childhood. Despite the established effectiveness of the pneumococcal polysaccharide vaccine in preventing pneumococcal pneumonia in adults, preliminary studies using an octavalent pneumococcal polysaccharide preparation have indicated that the vaccine is only marginally effective in preventing otitis media in infants. Recent studies have demonstrated that local secretory IgA antibody is secreted by goblet cells lining the middle ear and upper Eustachian tube and it is now evident that certain bacterial species are capable of inducing a local antibody response. We have sought to take advantage of the temperature differential in the respiratory tract and have selected mutants of S. pneumoniae which grow vigorously at 32°C, the temperature of the upper respiratory tract, but which do not replicate efficiently at the temperature of the lower respiratory tract, 37-38°C. Mutants of this type would be able to replicate efficiently in the nasopharynx and stimulate the production of local antibody but would not retain the capacity to produce disease in the lower respiratory tract because of their failure to grow to high titer at the restrictive temperature.

Progress

Following exposure of ts+ types I *S. pneumoniae* (PnIF strain) to nitrosoguanidine, 13 ts mutants were selected which were restricted in capacity to form colonies on blood agar at 38°C. While colony formation by the ts+ strain was unaffected over a broad range of temperatures (32-39°C), colony formation by ts mutants was altered or inhibited at temperatures ranging from 36°-39°C. *In vitro* studies indicate that the capacity to produce immunochemically reactive type I capsular polysaccharide is retained by ts mutants grown at permissive temperatures. When administered intra-peritoneally to mice (mean core temperature 37.6 ± 0.3°C) the ts+ strain was highly lethal ($LD_{50} = 10^{0.2}$ organisms) whereas 11 of the 13 ts mutants were markedly less virulent ($LD_{50} = 10^{5}-10^{7}$ organisms). In addition, mice immunized with ts mutants were resistant to subsequent lethal challenge with ts+ type I organisms. Examination of the *in vitro* growth curves of three selected ts mutants (ts-1, 3 and 4) suggest that attenuation may be related in part to altered growth rate and/or decreased survival time of ts organisms at restrictive temperatures. Studies are underway to determine whether polysaccharide synthesis is separately affected at restrictive temperatures. Following intraperitoneal inoculation of ts-1 (38°C shutoff temperature), ts-3 (36°C shutoff temperature), ts-4 (37°C shutoff temperature) or ts+ organisms, golden Syrian hamsters (mean core temperature 37.0 ± 0.6°C) were monitored for bacteremia, serum antibody response and evidence of disease. ts+ *S. pneumoniae* grew readily in the blood of hamsters attaining peak concentrations in 24 to 48 hours, a time associated with the development of pathological changes, illness and death. In contrast to the ts+ strain, the three ts mutants were found to be severely restricted in growth *in vivo*. Clearing of circulating ts organisms from the blood occurred rapidly after inoculation and was associated with the development of serum antibodies in 2-3 days. Measurable levels of antibody and protection from lethal challenge persisted at least 4-6 weeks in the animals inoculated with the ts mutants. Despite the isolation of apparent ts+ revertants from blood of animals receiving two of the three ts mutants (ts-1 and ts-4), illness and death were not observed. Revertants of ts-3 were not detected following growth *in vivo*.

Significance of the Program to the Institute

Pneumococcal infection is an important cause of respiratory tract illness, central nervous system disease, and otitis media with subsequent hearing loss in young children. Although polysaccharide vaccine appears to be highly effective in preventing pneumococcal pneumonia there is evidence that parenteral administration of a polysaccharide vaccine has considerably less effect in preventing otitis media. For this reason there is a need to explore alternate approaches to prevention of this type of ear disease.

Proposed Course of Study

We plan to study the complete set of ts mutants of *S. pneumoniae* in hamsters and mice utilizing the nasopharyngeal route of immunization and challenge. The capacity of the ts mutants to be transformed by DNA from wild type *S. pneumoniae* of type I or other serotypes will be investigated.

Keyword Descriptors

Streptococcus pneumoniae
Bacterial genetics

Publications

None

LI

RML

LSD

LBV

LMI

LPD

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LABORATORY OF VIRAL DISEASES

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PHS-NIH
SUMMARY REPORT

ANNUAL REPORT OF THE LABORATORY OF VIRAL DISEASES, NIAID

July 1, 1974 - June 30, 1975

Dr. Wallace P. Rowe
Chief, Laboratory of Viral Diseases

The program of LVD has three major long-term areas - mouse C-type viruses, host defense mechanisms, and papovaviruses - as well as a number of smaller projects. In general, good progress has been made.

The work of most immediate public health importance has come out of the research on interferon. The ribonuclease-resistant complex of poly I:poly C with poly L-lysine and carboxymethylcellulose, developed by Dr. Hilton Levy, is showing great promise as an interferon-inducing antiviral agent. Extensive collaborative studies in monkey model systems have now shown that it is highly effective for prophylaxis against the highly fatal disease simian hemorrhagic fever and against herpetic keratoconjunctivitis, and for early post-infection treatment of yellow fever and rabies street virus. Aerosol administration in monkeys produces high levels of interferon in the lungs without any evidence of toxicity. Initial clinical trials in man will be started in the near future. There is a very real possibility that this interferon inducer could represent a major breakthrough in the control of viral infections.

The interferon program will suffer a great loss in that Dr. Baron is leaving NIH to become Chairman, Department of Microbiology, University of Texas Medical Branch at Galveston, Texas.

The LVD program on murine C-type viruses plays a very important role in the scientific community. C-type viruses are the subject of the most intensive investigation of any virus group, being the focus of most of the Virus Cancer Program of the National Cancer Institute. It appears to us that much of this work is predicated on a simple cause-effect relationship, and it is our feeling that a major function of our program, which is an examination of one system in depth, is to challenge this assumption and to provide insights into the complexity of the virology and the host-virus relationships of C-type virus infection.

Now that it is well established that the genome of murine leukemia viruses (MuLV) are carried as chromosomal loci in mice, increasing attention is being devoted to the question of the evolution of these loci. By means of biochemical probes for viral gene sequences in cellular DNA, we can now analyse mice and other rodents for the presence of several distinct classes of viral gene sequences. Some are group specific, in the sense that they are common to all C-type viruses of mice, while the remainder are "type specific" in that they appear to be unique to each of the 3 or 4 major biological classes of MuLV. An intriguing pattern appears to be emerging. The group specific sequences are present, in multiple copies, in all mice, both laboratory strains and feral. In contrast, some of the type-specific sequences, and the viruses to which they correspond, are not found in all mice, but only in certain inbred strain or certain demes of wild mice. This must mean that the evolution of the different classes of sequences is different, and suggests that the different classes of virus may arise as recombinants between sequences of exogenous virus from some other species and the group specific sequences of the endogenous MuLV. It is hoped that hybridization studies of the phylogenetic relatives of *Mus musculus* may clarify this point.

In the field of papovavirus infection, recent LVD research has pointed up several major areas of interest. The association of BK virus infection with the Wiscott Aldrich immunodeficiency syndrome, observed initially by Dr. Takemoto, has been confirmed, although the cause-effect relationship is not yet clear. The integrated study of serologic and nucleic acid sequence relatedness between BK and SV40 viruses has pointed up a major discrepancy in the current picture of the genetic map of SV40; these two viruses appear to have identical T antigens, yet show no sequence homology in that portion of the SV40 genome which Dr. Lewis's studies with nondefective adeno-SV40 hybrids have shown to contain the genes for T-antigen induction. This finding, together with Dr. Lewis's recent finding that this T-antigen genetic region appears to be associated with oncogenicity, further points up that there are important biological questions concerning the genetic coding for T antigen and its relationship to oncogenic transformation. There appears to be a distinct possibility that T antigen is a protein consisting of a virus-coded polypeptide attached to a host protein. The nature of this host protein and the change in its function resulting from acquisition of viral sequences could be a key to understanding oncogenesis by DNA viruses.

The problem of needing biohazard containment for working with nondefective adeno-SV40 viruses continues to hamper these studies. Dr. Lewis has had the use of facilities in Bldg. 41 for several months, and has been able to prepare fresh virus stocks to be used for biochemical and oncogenesis studies with inactivated virus. However, the need for a long term solution to the containment problem is urgently needed.

LVD scientists received two types of recognition during the past year. Dr. Rowe was elected to membership in the National Academy of Sciences, and Dr. Baron was appointed to the Editorial Advisory Board of *Archives of Virology*.

Interferon therapy in primates. New interferon-inducer effective against viral infections of monkeys. The poly I:poly C, poly L-lysine, carboxymethylcellulose complex is an effective inducer of interferon in monkeys and chimpanzees, while plain poly I:poly C is not. The new complex has been effective in monkeys in controlling street rabies, yellow fever, herpes keratoconjunctivitis. and Simian hemorrhagic fever. The complex is being tried in man (Levy, Buckler).

Alterations in noninfected cells induced by interferon. It has been found that interferon treatment of uninfected cells leads to modifications in polysome associated cellular mRNA and tRNA. It is suggested that these alterations are related to the way by which cells recognize and distinguish between viral RNA and cell RNA, translating the latter, but only translating the former to a limited extent (Levy, Riley, Lvovsky).

Interferon as a possible regulator of immunity. Studies to determine which subpopulation of mouse spleen cells was responsible for interferon production after mitogen stimulation suggested that peripheralized T cells are required. The amount of interferon produced under mitogen or antigen stimulation was shown to be sufficient to markedly inhibit the primary *in vitro* plaque forming cell response to both T dependent and T independent antigens. These findings raise the possibility that during immunological sensitization of an animal to antigen, the antibody response can be limited when sensitized T cells react with antigen and produce interferon (Baron, Green, Dianzani).

Role of cell-bound interferon. Previous studies by others indicated that 1% of applied interferon was bound to cells and might be the initial step leading to antiviral activity. The present studies provided evidence indicating that the cell-bound interferon did not act in this manner but served as a reservoir which after washing away of unbound interferon, replenished the extracellular interferon at low concentrations. Thus, undefined are the cell-reacting fraction of interferon and the required time of contact of interferon with the cell (Baron, Green, Dianzani).

Late interferon therapy of viral infected cells. Treatment of cells in culture with high concentrations of interferon at different times after viral infection demonstrated that interferon lost its effectiveness when applied one to three hours after initiation of infection. Thus, interferon may act to protect cells recently infected and also cells not yet infected with these viruses but not cells infected more than several hours (Baron, Green, Dianzani).

Relationship of recurrent herpes simplex virus infections in man to virus growth and interferon activity in human gingival cultures. No correlation was found between history of recurrent herpetic infections and interferon production or susceptibility of the patient's cultured gingival cells to herpes simplex virus infection. The number of patients was too small to rule out a possible inverse relationship between interferon sensitivity and a clinical history of recurrent disease (Baron, Green, Dianzani).

Biochemical detection of murine leukemia virus (MuLV) sequences in cell DNA. Nucleic acid hybridization studies using an AKR-MuLV DNA probe and cellular DNA obtained from mice segregating for one of the virus-inducing genes (*Akv-1*) present in AKR mice have provided proof that this locus actually contains virus genetic material. Complete correlation of AKR viral DNA sequences and the presence of *Akv-1* was obtained (Chattopadhyay, Rowe).

An allele of the Fv-1^{n} *locus affects sensitivity of cells to N-tropic MuLV*. Cells derived from certain *Fv-1*^{n} mouse strains are less sensitive to N-tropic MuLV than others. It has been found that this property is controlled by an allele of the *Fv-1*^{n} gene locus which is present in 129, RF, NZB and NZW strains and is segregating in NIH Swiss, General Purpose Swiss and HSFS/N inbred NIH Swiss mice. In addition to affecting exogenous virus infection this allele markedly restricts expression of genetically transmitted virus (Rowe, Hartley).

Genetic transmission of N- and B-tropic virus in B10.Br/Li mice. Tests of a subline of the congenic B10.Br mouse strain for induction of N- and B-tropic virus MuLV by IUdR indicate that this strain, although long inbred, is segregating for loci controlling production of virus, suggesting a recent insertion of viral genomes. Progeny testing indicates that two independent loci for induction of N-tropic virus are present, one of which is also associated with B-tropic virus induction (Rowe, Hartley).

Cytotoxic activity of Abelson virus-induced lymphoma cells. Cells from B-cell lymphomas induced by Abelson virus are cytotoxic for normal mouse cells *in vitro*, but not for mouse cells infected with MuLV's of the same virus interference group as Abelson virus. This is a unique finding, since lymphocyte cytotoxicity is seen with T cells, and rarely with other classes of lymphocytes (Sklar, Rowe).

Effects of interferon on MuLV infection. Interferon has been shown to have an inhibitory effect on acute infection, chronic production, and endogenous activation of MuLV, but only while present in the system. Interferon does not affect viral protein or RNA synthesis, but apparently inhibits viral assembly at the cell membrane (Pitha, Rowe, Staal).

A new class of murine leukemia viruses, the amphitropic viruses. In the course of studying MuLV isolated from wild mice trapped in the Los Angeles area it was found that several isolates could be separated into two populations by limiting dilution titration procedures. One population is N-tropic virus with host range restricted to mouse and rat cells; viruses in this category are serologically distinguishable from the mouse-tropic viruses of inbred mice but one in the same class by viral interference studies. The second population can replicate in N-type mouse cells and also in cells of many heterologous species, including most but not all of those sensitive to xenotropic MuLV. These viruses, termed "amphitropic", are serologically distinct from mouse- and xeno-tropic viruses and represent a third interference class (Hartley, Rowe).

Isolation of papovaviruses from urine of Wiskott-Aldrich patients. A total of 3 new isolates of papovaviruses have been made from the urine of patients with the Wiskott-Aldrich syndrome, an immunodeficiency disease primarily affecting cellular immunity. The isolates are closely related to the prototype BK virus. The urine from one patient has been periodically examined and virus has consistently been detected or isolated from his urine. Thus, patients with this disease appear to excrete virus constantly, unlike normal individuals from whom this virus has never been isolated (Takemoto, Mullarkey, Mason).

Biological and biochemical characterization of human papovaviruses. The two related but serologically distinct human papovaviruses, BK and JC, share common virion (V) as well as tumor (T), antigens; these antigens are also shared with the Simian virus 40. However, the degree of relatedness among the 3 viruses as determined by nucleic acid hybridization studies is only 10 to 20%. Using SV40 fragments cleaved by restriction endonuclease in hybridization experiments with BK DNA, only fragments assigned to the "late" region of the SV40 map were found to share homology with BK DNA. Since no homology was detected in the "early" fragments, this finding raises questions concerning one of the early gene products, the T-antigen, which has been thought to be virus-coded. Since the BK and SV40 T-antigens cannot be distinguished, the lack of homology in the "early" regions of the viral genomes of the 2 viruses may mean that T-antigens may be largely cellular proteins, partly virus specified (Takemoto, Mullarkey, Mason). (Studies done in collaboration with Drs. Khoury, Martin, and Howley, LBV).

Growth of JC virus. The JC virus (implicated in at least 15 cases of PML disease) has been grown in sufficient quantities to do studies on its characterization. The virus is severely restricted in its host range, and can only be grown in human fetal brain cells. Replication is extremely slow, requiring 3 to 6 weeks for maximum virus production. Unlike BK, the JC virus is highly oncogenic for hamsters and produces a variety of tumors when injected intracerebrally, intraperitoneally, and subcutaneously (Takemoto, Mullarkey, Mason).

Transformation by BK. Hamster cells have been successfully transformed by BK viral DNA using the calcium phosphate precipitation technique. The cells are malignant, inducing progressively growing tumors. An unusual finding in tumor-bearing animals was the presence of anti-nuclear antibody which reacts against all normal cells tested. The significance and implications of this finding await further study, but this provides a unique system for studying the development and consequences of autoantibodies (Takemoto, Mullarkey, Mason).

Comparative efficiency of transformation of hamster and rat cells by Ad2 and the nondefective hybrids. The two nondefective Ad2-SV40 hybrids with the largest segment of SV40 DNA (48% of the SV40 genome) transform hamster cells 6 to 10 fold more efficiently than nonhybrid Ad2. The Ad2$^+$ND$_2$ hybrid containing 32% of the same region of SV40 DNA transforms hamster cells no more efficiently than Ad2. Each of these hybrids transforms rat cells more efficiently than nonhybrid Ad2. These data suggest that the acquisition of certain segments of the SV40 genome can enhance the transforming ability of nonhybrid Ad2 and that the mechanism of transformation may differ in hamster and rat cells (Lewis).

Induction of tumors in hamsters with Ad2 transformed cell lines. Most of the cell lines established from transformed foci in the quantitative transformation assays produce tumors when injected into newborn hamsters. When tumors developing in newborns, however, are transplanted to 21 day old hamsters the majority regress after becoming 10 to 20 mm in diameter. Multiple passages of tumors in newborn hamsters before transplantation into weanlings does not alter tumor regression in the 21 day olds. It appears that the maturation of the hamsters immune system is responsible for the rejection of these tumors. The regression phenomenon can be overcome with tumors induced by certain cell lines by massive inoculation of cells (Lewis).

Heteroduplex mapping studies on the B55 hybrid. The B55 hybrid pool is the virus stock from which all 5 nondefective Ad2-SV40 hybrids were isolated. The Ad2$^+$ND$_4$ hybrid was isolated from the B55 hybrid by plaque studies in monkey cells; this hybrid is biologically indistinguishable from B55. Comparing the DNA of B55 and Ad2$^+$ND$_4$ by electron microscopy no major differences could be detected in the amount of Ad2 deleted (4.5% of the Ad2 genome) or the size of the SV40 inserted (48% of the SV40 genome) in the B55 hybrid DNA [Lewis, Kelly (Johns Hopkins)].

Induction of tumors in newborn hamsters with UV inactivated $Ad2^+ND_4$ and B55. After 150 to 180 days 5-10% of hamsters injected as newborns with UV inactivated $Ad2^+ND_4$ or B55 have developed tumors. No tumors have developed in hamsters injected with 2 strains of Ad2 or $Ad2^+ND_2$. Hamsters carrying tumors induced by the $Ad2^+ND_4$ hybrid developed antibodies to both SV40 and Ad2 T antigens. Since 2 strains of UV inactivated nonhybrid Ad2 and the $Ad2^+ND_2$ have not produced tumors after 270 days, these studies associate the induction of tumors in hamsters with that region of the SV40 genome lying between the end of the SV40 segment in $Ad2^+ND_2$ at 0.43 SV40 units and the end of the SV40 segment in the $Ad2^+ND_4$ hybrid at 0.59 SV40 units (Lewis).

Effect of intraperitoneal silica on virus infection of macrophages. Contrary to previous reports that silica decreased macrophages *in vivo*, it was found to induce proliferation of these cells *in vivo*, thus aggravating the chronic viremia of HSV and LDV (duBuy).

Further evidence for lysogenicity of amebal viruses in Entamoeba histolytica. Clones of the ABRM strain of E. histolytica, as wild type, produce both filamentous and icosahedral viruses. These observations are compatible, along with previous data, with this amebal strain being doubly lysogenic (Mattern, Daniel, Kontonis).

Similarity of filamentous amebal virus to certain bacterial viruses. The filamentous amebal virus resembles the 7 mm diameter filamentous bacterial viruses in morphology and appears to similarly band at 1.29 density in CsCl (Mattern, Daniel, Kontonis).

Universal AAV-sera. The discovery that polypeptide sera prepared against one type of AAV will react against all sera types of AAV in cell culture by immunofluorescence makes it possible to produce a universal sera for detecting AAV from all sources (Hoggan).

Isolation of a paramyxovirus-like virus from wild mice. A virus with many properties of paramyxoviruses has been detected as a latent infection of the kidneys of wild house mice. The virus is serologically distinct from known ortho- and para-myxoviruses and does not possess neuraminidase (Manders, Hartley, Rowe).

Project No. Z01 AI 00010-13 LVD
1. Viral Diseases
2. Viral Oncology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Immunologic and Other Studies of Animal Leukemia Viruses.

Previous Serial Number: NIAID 68H

Principal Investigator: Dr. M. K. Cook.

Other Investigators: Dr. W. P. Rowe, Miss Johnna Sears.

Cooperating Units: None.

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	24/12	0	0	24/12
Professional:	12/12	0	0	12/12
Other:	12/12	0	0	12/12

Project Description:

Objectives: Definition of the immunology, biology, and natural history of viral agents associated with Marek's disease and the leukemias of fowl, guinea pigs, hamsters, and cattle.

Methods Employed: Immunofluorescence, agar-gel precipitation, immunoelectrophoresis, radio-immunoassays, neutralization tests and complement-fixation tests were employed to characterize the antigenic components of the viruses and the antibody responses to these components in various animals. Gradient centrifugation, column chromatography and polyacrylamide gel electrophoresis were used to separate the antigenic components of guinea pig and hamster herpesviruses. Tissue culture systems and standard animal inoculation procedures were used.

Major Findings: Considerable interest has centered on the role of multiple infection with C-type particles and herpesvirus in the production of Marek's disease and leukemia in fowl and the transformation of cells in tissue culture. As yet, this question has not been resolved in a satisfactory manner using an avian system. The previous studies were hampered partially by technical problems involving lack of inbred chickens, failure to obtain high infectivity titers of Marek's herpesvirus, and the instability of the cell-free virus and its antigenic components. These factors prompted us to examine other systems to study the role of multiple infections and oncogensis. It was hoped also that production of higher-titered material would allow us to more easily separate antigenic differences in virus strains which could then be identified with immunologic conversions of viruses after laboratory adaptation. For this reason high oncogenic and low-oncogenic or non-oncogenic strains of guinea pig herpesviruses were obtained from Dr. Hsuing. The infectivity titers of the guinea pig viruses were reported to be 100-1,000X those obtained with the chicken herpesvirus, and the viruses were said to be considerably more stable. Furthermore, C-type viruses were reported to be associated with all the guinea pig virus strains studied. In our laboratory, we were not able to confirm the presence of C-particles or C-type viruses in any of the strains studied. Only herpes-type viruses were observed. In addition, inoculation with high-titered virus did not produce leukemia or other oncogenic changes in any of the many strains of guinea pigs used. No differences were found in the type of cytopathic changes produced in guinea pig tissue cultures infected with either high or low or non-oncogenic strains of virus. Gs antigens produced by both "high" and "non-oncogenic" guinea pig herpesvirus were produced, concentrated, and partially purified. Polyacrylamide gel electrophoresis of the antigens from these strains failed to demonstrate qualitative differences in them. The gs antigens from both "high" and "low" oncogenic strains of guinea pig herpes virus failed to produce antibody in guinea pigs. Antibody responses were elicited, however, in hamsters and rabbits. This may be related to failure of the guinea pig herpesvirus strains used to produce herpesvirus "A" antigens.

Significance to Biomedical Research and the Program of the Institute: The development of suitable, sensitive, laboratory systems should help in the study of the possible interactions of DNA and RNA viruses implicated in oncogenesis.

Further information is required from other animal systems to determine if the immunologic conversions of virus strains noted after laboratory adaptation of pathogenic Marek's disease herpesvirus strains do, indeed, have meaning in relation to tumor production and lympho-proliferative disease, mechanisms of cellular immunity, production of spontaneous tumor regression, or hypersensitivity reactions.

Proposed Course of the Project: As the guinea pig herpesvirus strains available to us proved to be completely unsatisfactory as a system for the study of the interaction of C-type viruses and herpesviruses, and, furthermore, failed to produce oncogenic changes in any strains of animals studied, or even transformation in tissue culture, this line of experimentation will be discontinued.

Emphasis will we placed on hamster embryo tissue culture systems as tools to examine the interactions of C-type viruses and herpesviruses. Hamster embryo cells treated with UV-irradiated Marek's disease herpesvirus, both in the presence or absence of C-particles, will be studied for transformation and persistence of the viral genome in the transformed cells which develop. If isolation and high-containment areas are made available, it may be possible to initiate studies with oncogenic and non-oncogenic simian herpesviruses and the C-type particles reportedly associated with them.

Hamster embryo cells seem to be extremely sensitive in undergoing morphological cellular transformation after treatment with herpesviruses. At least seven species (man, mice, rats, guinea pigs, cattle, monkeys, and chickens) and the hamster itself harbor C-type particles. We will attempt to study the peculiarities of the hamster tissue culture cells and the role, if any, of the C-particles present in transformed cells.

Key Descriptors: Herpesvirus, Marek's disease, gs antigens, transformation, guinea pig herpesvirus, C-particles, hypersensitivity, leukemia, viral interactions.

Project No. Z01 AI 00011-10 LVD
1. Viral Diseases
2. Viral Oncology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies of the Small, Highly Resistant DNA Containing Viruses Belonging to the Family Parvoviridae

Previous Serial Number: NIAID 71A

Principal Investigator: Dr. M. David Hoggan

Other Investigators: Dr. Aurobindo Roy and Gunter F. Thomas

Cooperating Units: Dr. Kenneth I. Berns, Johns Hopkins University School of Medicine, Baltimore, Maryland; Dr. Neil L. Blacklow, Department of Infectious Diseases, Boston University Medical School, Boston, Massachusetts

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	42/12	0	0	42/12
Professional:	12/12	0	6/12	18/12
Other:	24/12	0	0	24/24

Project Description:

Objectives: To study the biological, immunological, biophysical and biochemical characteristics of the parvoviruses in order to further our understanding of the basic mechanisms of parvovirus replication and persistence, thus gaining an insight to their role in the diseases of various hosts including man. To develop more efficient methods of detection and identification of these agents, as well as test various procedures for elimination of these agents from their hosts and the environment.

Methods Employed: Various parvoviruses from widely divergent phylogenetic hosts are grown in their homologous host cells with various radioactive labels.

The labeled virus is purified by treatment with various enzymes and isopycnic centrifugation in CsCl gradients. After treatment with appropriate antiserum to further reduce cell and helper virus contamination, the virus is rebanded in CsCl.

The viruses are disrupted then separated into their constituent polypeptides by polyacrylamide gel electrophoresis. The individual polypeptides are then characterized and antisera prepared in guinea pigs. As new parvoviruses are isolated they are grown in appropriate host cells then purified and specific antiserum prepared in laboratory animals.

Because some of the isolates replicate only to very low titers it has been helpful to use immunoelectronmicroscopy for some of our comparative serological studies.

As previously reported the technique of establishing AAV-carrier cultures in the absence of helper provides a model for studying their persistence in nature. Such occult "lysogenic" cultures can be induced to produce virus, as measured by complement fixation, immunofluorescence (FA) and electronmicroscopy, when challenged with helper adenovirus. DNA/DNA reassociation kinetics using hydroxyapatite for the detection of single stranded DNA are used for detecting AAV-DNA in the "lysogenic" carrier cultures.

UV inactivation as well as various chemical inhibitors are tested in vitro and in cell culture by standard procedures for their effectiveness in eliminating these agents.

Major Findings: Because of a world wide need for good standard reagents for the various parvoviruses including the AAV's the Research Resources Branch of our institute has attempted during the last 3-4 years to have AAV reagents produced under contract. However, because of the difficulty of working with these reagents such endeavors have largely failed. Except for the production of small pools of crude virus seed and a limited amount of guinea pig anti AAV-3 sera no other AAV reagents were produced using the normal contract mechanism. In order to complete the production of the proposed AAV-1 through 4 sera, the personnel of this project because of their past experience and need for such reagents consented to produce and purify the required antigens and produce the anti AAV-1, 2 and 4 sera.

Approximately 20,000 CF units (about 10^{14} infectious doses) of each of the AAVs was grown in cell culture and purified for use as immunogen. A small contract for 150 AAV, adenovirus free guinea pigs including animal care and housing with limited access was let with George Washington University School of Medicine Animal Resources Branch. After being pretested each animal was injected with 400 CF units of AAV antigen plus complete Freund's adjuvant then serially bled 4 times over a 60 day period. Thus far 500 ml each of AAV-1 and AAV-2 guinea pig antisera has been produced. The sera have titers greater than 1280 in CF against their homologous antigens and no measurable titer when tested against helper adenovirus. These two serum pools are currently under test for their FA and serum neutralization titers. AAV-4 antigen has been prepared, purified and the animals injected. The first two bleedings have been accomplished. The AAV-4 pool will be completed by the end of the current fiscal year. These reagents should be packaged, independently tested, and ready for distribution by the Research Resources Branch early next year.

We previously reported no cross reactivities between the human, the simian and the canine AAV's when tested by conventional serological tests using hyperimmune serum produced against whole purified virions in guinea pigs. This finding has now been extended to the avian and bovine AAV-isolates. Similarly, no cross reactivity between 6 independently replicating parvoviruses and the AAV's was found. A recent finding of practical importance is the fact that antisera made against the individual or mixed polypeptides of a single AAV type will not cross react with any of the independent replicating parvoviruses but will react with any AAV regardless of type or species of origin when tested by immunofluorescence.

Our recent studies have indicated that the AAV's are extremely sensitive to UV. Exposure to two GE germicidal bulbs at 27.5 cm (60 ergs/mm^2/sec) for one minute resulted in inactivation of greater than 10^6 TCID50's of AAV-1 and greater than 10^5 TCID50's of AAV-2.

Preliminary attempts to demonstrate that the AAV-carrier state developed in D-6 cells is non-episomal in nature have been completed. These experiments were carried out based on the long established phenomenon that bacterial episomes when in the autonomous state can be eliminated by treatment of the host bacteria with acridine dyes. When bacterial episomes are in the integrated state the acridine dyes have no effect. When AAV-carrier cells were treated with acridine orange under the appropriate conditions there was no reduction in the amount of inducible AAV. This is suggestive evidence that the AAV genome is integrated into the host DNA.

New clones of AAV-2 carrier cells have been established and characterized. They produce between 5-10 x 10^3 TCID50's and 4 x 10^{-3} CF antigen units of AAV per cell when challenged with appropriate helper. Unhelped cultures carry 3 to 5 genome equivalents of AAV per diploid host genome.

Collaborative studies have indicated that purified AAV in the absence of any helper virus can effectively reduce the oncogenic expression of HSV transformed cells in hamsters.

Significance to Biomedical Research and the Program of the Institute: In the ten to fifteen years since their discovery and characterization as a virus group, the parvoviruses have been found in increasing numbers from a wide variety of phylogenetic hosts. They have been found to cause a number of different disease syndromes in nature as well as in the laboratory. They also frequently appear as passenger or contaminating viruses causing confusion and rendering much experimental data difficult if not impossible to interpret.

The completion of AAV-reference reagents should be of inestimable value to many laboratories who are carrying out research on the adenoviruses or Ad-SV40 hybrid viruses as well as the increasing number of investigators interested in the parvoviruses. The finding that AAV polypeptide antisera from one AAV can be used to detect AAV-replication of any type is especially important since it will allow for the detection in cell culture of new yet undiscovered AAV's.

The finding that the parvoviruses are extremely sensitive to U.V. inactivation not only offers hope of finding a simple rather inexpensive method of ridding the laboratory environs of these extremely chemically resistant viruses but offers a great potential for preparing effective killed vaccines for such diseases as hepatitis type A of humans or such monetarily important animal diseases as panleukopenia of cats or aleutian mink disease.

The finding that the parvoviruses can persistently infect their hosts cells in a presumbably integrated state without any overt evidence of infection suggests an important mode of survival with a vertical transmission of these agents. The finding that AAV not only reduces the oncogenic potential of the oncogenic adenoviruses but also can function to alter the cancer expression of HSV transformed cells including reduction of metastasis in vivo is of great interest and importance in understanding the oncogenic processes.

Proposed Course of the Project: Studies designed to elucidate the interrelationships between helper, host and parvovirus protein synthesis will continue.

Comparative studies between various parvoviruses, their relationship to natural and laboratory diseases will continue.

Using the carrier state systems with various TS mutant helper viruses now available we will study the role of various helper virus functions in expressing AAV reproduction.

Key Descriptors: Parvoviruses, adeno-associated virus (AAV), nucleic acid hybridization, immune electron microscopy, carrier-culture systems, virus-helper interactions, polypeptide antibody, virus inactivation.

Project No. Z01 AI 00012-13 LVD
1. Viral Diseases
2. Viral Oncology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies of Mouse Leukemia Viruses

Previous Serial Number: NIAID/NCI 71D

Principal Investigators: Dr. Janet W. Hartley, LVD, NIAID
Dr. Wallace P. Rowe, LVD, NIAID

Other Investigators: Dr. Sisir K. Chattopadhyay, LVD, NIAID
Dr. Stephen Staal, LVD, NIAID
Dr. Akinori Ishimoto, LVD, NIAID (from Sept. 1974)
Dr. Ernest Manders, LVD, NIAID
Dr. Rex Risser, LVD, NIAID (from Feb. 1975)

Cooperating Units: Inside NIH:

Dr. Tadao Aoki, VLL, NCI
Dr. Ronald Herberman, LCBGY, NCI
Dr. Robert Huebner, VC, NCI
Dr. Arthur S. Levine, DCT, NCI
Dr. Wade Parks, VC, NCI
Dr. Michael Potter, LCBGY, NCI
Dr. Edward Scolnick, VLL, NCI

Outside NIH:

Dr. Edward A. Boyse, Sloan Kettering Institute for Cancer Research, New York, New York
Dr. Theodore Bremner, Howard University, Washington, D.C.
Dr. H. Ikeda, Sloan Kettering Institute for Cancer Research, New York, New York
Dr. Frank Lilly, Albert Einstein College of Medicine, New York, New York
Dr. Douglas R. Lowy, Yale University School of Medicine, New Haven, Connecticut
Dr. Michael N. Oxman, Children's Hospital Medical Center, Boston, Massachusetts
Dr. John C. Parker, Microbiological Associates, Inc., Bethesda, Maryland
Dr. Paula Pitha, Johns Hopkins University, Baltimore, Maryland
Dr. Marshall D. Sklar, Stanford University Hospital, Stanford, California
Dr. E. Stockert, Sloan Kettering Institute for Cancer Research, New York, New York

Project No. Z01 AI 00012-13 LVD

Man Years:

Total:	12.75
Professional:	4.0
Other:	8.75

Project Description:

Objectives: To utilize sensitive procedures for genome detection and isolation and quantitation of infectious virus in studying the natural history of murine leukemia virus (MuLV) infection.

Methods Employed: Tissue culture procedures, using plaque and transformation assays; tests for antigen induction by complement fixation and fluorescent antibody procedures; reverse transcriptase assays. Mouse inbreeding, to select for specific virus-inducing or virus-related genes. Starch-gel electrophoresis for assays for marker isoenzyme loci. Nucleic acid hybridization techniques.

Major Findings:

Biochemical studies of MuLV infection: In studies of the viral nucleic acid sequences of mouse-tropic AKR-type murine leukemia virus detectable in high-, low-, and non-virus-yielding mouse strains, it was found that the complete AKR virus genome is present in the DNA of high-virus-yielding strains (AKR, C$_3$H/Fg) and low-virus-yielding strains (BALB/c, DBA/2, C$_3$H/He, and C$_{57}$Bl/6), while the DNA of non-virus-yielding mice (NIH Swiss, 129/J, C$_{57}$L/J and NZB/N) contains only a part of the genome. In strains with the complete genome, the reassociation rates of the DNA's with the single-stranded AKR viral DNA probe (complementary to AKR viral RNA) indicate two populations of virus-specific DNA sequences, one more abundant and one less abundant. The DNA from low-virus-yielding strains contains fewer copies of the less abundant species while that from non-virus-yielding strains lacks a major portion of the viral genome, apparently corresponding to the less abundant set of sequences.

Proof that one mouse-tropic virus inducing locus in AKR mice, Akv-1, contains viral genetic material was obtained by demonstrating that AKR-type viral DNA sequences correlated completely with presence of Akv-1 in mice segregating for this locus.

Studies of the Fv-1^n locus: In the original studies describing the genetic basis of resistance of cells from various mouse strains to exogenous murine leukemia virus, it was noted that among Fv-1^n mouse strains (i.e., strains sensitive to N-tropic virus but markedly resistant to B-tropic virus) some showed decreased sensitivity to N-tropic virus. It now appears that this property is controlled by an allele of the Fv-1^n gene locus, is found in the 129, RF, NZB, and NZW strains, and is apparently segregating in NIH Swiss, General Purpose Swiss, and the HSFS/N inbred NIH Swiss line. This poorly permissive Fv-1^n allele is able to restrict growth of genetically transmitted

N-tropic virus in hybrid mice almost as effectively as Fv-1^b, and consequently is a major variable in infectious expression of Gross-AKR virus.

Genetics of N- and B-tropic virus induction in B10.Br mice: The congenic mouse strain B10.Br/Li is a B-type mouse which demonstrates a high frequency of B-tropic virus in vivo; IdU induction of tissue culture cells results in a high frequency of N-tropic virus and generally B-tropic virus production as well. Preliminary studies of backcross progeny indicate that B10.Br possesses two independent loci for induction of N-tropic virus, one of which is also associated with B-tropic virus induction. Although B10.Br has been inbred for many years, the mice are not homozygous for these loci; this strain may represent an instance where new insertions of C-type viral genomes into the germ line has taken place in the past few years. It is also exceptional in being the only strain from which B-tropic virus can be induced by IUDR treatment.

Detection of a new class of murine leukemia viruses: When it was found that MuLV isolates from Los Angeles area feral mice were capable of infecting heterologous species cells, limiting dilution purification studies were carried out in attempts to resolve possible mixtures. It has been found that several isolates of feral MuLV contain two components, an N-tropic virus whose host range is restricted to mouse and rat cells, and a virus population, termed "amphitropic", which can propagate in N-type mouse cells and in a wide variety of heterologous species cells. The heterologous cell host range of amphitropic virus is similar to that of xenotropic viruses except that it does not infect duck or quail cells. Using MSV pseudotypes of mouse-tropic, xenotropic, and amphitropic MuLV, it has been found that the three host-range classes can be distinquished on the basis of interference activity, i.e., that representatives of each class do not significantly interfere with each other.

Assay systems for xenotropic viruses: The commonly used assays for xenotropic virus have been by MSV rescue or determination of reverse transcriptase activity; neither method is satisfactory for direct quantitation of xenotropic MuLV yields. We have found that quantitation is possible by enumeration of foci of fluorescent cells in cultures infected with dilutions of xenotropic virus; acetone-fixed preparations are stained with a direct test with fluorecein-conjugated anti-Moloney serum (obtained from Dr. Roger Wilsnack, Huntingdon Research Center). Using this test, several cell systems, including human, rabbit (SIRC), and mink have been compared for sensitivity to a number of xenotropic MuLV. The results of FA focus assays in SIRC cells correlate well with assays in the feline S+L- line recently described by others, while FA assays in mink lung cells have been consistently superior in sensitivity, particularly for the virus released by NZB cell lines which is poorly infectious for other cell lines tested.

Cytotoxicity of B-cell lymphoma cells. Lymphoma cells of Abelson-virus induced tumors, which are non-thymic in origin, have been found to be intensely cytotoxic for normal mouse cells. Of other species cells tested, only rat cells are affected by the cytotoxicity, and this is much less than with mouse cells.

Mouse cells are protected from the cytotoxicity if they are infected with leukemia virus of the same interference group as that produced by the lymphoma cells.

Effect of interferon on C-type virus infection: While it has been known for some time that C-type viruses are sensitive to inhibition by interferon, it is now clear that there are highly unique features of this inhibition. The interferon effect is seen on exogenous infection, endogenous virus activation, and chronic virus production; in all instances it is transitory, virus production being inhibited only while interferon is present. The effect does not appear to be on synthesis of viral protein or RNA, but appears to involve virus assembly at the cell membrane. It is possible that synthesis of some crucial assembly protein is inhibited, while that of the major virion proteins is not; this would be a unique system also, in that the effect of interferon on other viruses is through inhibition of all viral proteins.

Isolation of a Paramyxovirus-like Virus from Wild Mice: During the course of trapping wild house mice for studying their leukemia virus infection patterns, a cytopathic virus was isolated from kidney tissue cultures. This virus shows many properties of paramyxoviruses but does not possess neuraminidase and is antigenically distinct from all known ortho- and para-myxoviruses. It is unique in being carried as a latent renal infection.

Significance to Biomedical Research and the Program of the Institute: The development of methods for virus detection, propagation, and quantitation is essential to studying the natural occurrence and role in tumorigenesis of the murine leukemia viruses. The quite recent recognition of the xenotropic and amphitropic viruses is an example of the continuing revelation of the complexities of murine leukemia virus infection and the incompleteness of present knowledge.

The application of precise genetic and biochemical methods to the study of integration of viral genetic information may permit the correlation of specific virus sequences with development of particular types of tumors. Comparative study of the genetic information specified by different classes of murine leukemia viruses in various strains of mice is now feasible and it may be possible to determine the evolutionary origin of MuLV infection.

Proposed Course: Studies will continue on localizing the viral integration sites in various strains of mice and on the genetic relationship of various markers of MuLV expression and control.

Biochemical studies will attempt to distinguish between viral sequences specified by different murine leukemia viruses in the DNA of mice with differing expression of MuLV information. DNA from other rodent species will be tested for homology with MuLV viral DNA probes.

We will attempt to classify mouse-tropic AKR-Gross, FMR, and feral isolates, xenotropic viruses, and amphitropic feral isolates by neutralization testing and, in collaboration with Dr. Wade Parks, by analysis of viral proteins.

The feral mouse-tropic and amphitropic isolates will be tested for ability to induce tumors and/or neurologic disease, in mice, in attempts to reproduce the unusual paralytic disease induced by certain feral MuLV isolates, as described by Gardner and Huebner.

The interaction of amphitropic viruses with mouse-tropic viruses will be studied in attempts to detect genetic recombination.

Studies of the ability of Abelson virus and other MuLV to transform lymphocytes and other cell types will be resumed.

Publications:

Chattopadhyay, S.K., Lowy, D.R., Teich, N.M., Levine, A.S., and Rowe, W.P.: Qualitative and quantitative studies of AKR-type murine-leukemia-virus (MLV) sequences in DNA of high-, low-, and non-virus-yielding mouse strains. Cold Spring Harbor Symposium Quant. Biol., 39: 1085-1101, 1974.

Chattopadhyay, S.K., Rowe, W.P., Teich, N.M., Lowy, D.R.: Definitive evidence that the murine C-type virus inducing locus Akv-1 is viral genetic material. Proc. Nat. Acad. Sci. U.S.A., 72: 906-910, 1975.

Hartley, J.W. and Rowe, W.P.: Clonal cell lines from a feral mouse embryo which lack host-range restrictions for murine leukemia viruses. Virology, 1974. In press.

Lilly, F., Duran-Reynals, M.L., and Rowe, W.P.: Correlation of early murine leukemia virus titer and H-2 type with spontaneous leukemia in mice of the BALB/c x AKR. J. Exp. Med., 141: 882-889, 1975.

Lowy, D.R., Chattopadhyay, S.K., Teich, N.M., Rowe, W.P., and Levine, A.S.: AKR murine leukemia virus genome: Frequency of sequences in DNA of high-, low-, and non-virus-yielding mouse strains. Proc. Nat. Acad. Sci., 71: 3555-3559, 1974.

Nomura, S., Dunn, K.J., Mattern, C.F.T., Hartley, J.W., and Fischinger, P.J.: Revertants of mouse cells transformed by murine sarcoma virus from a clone of BALB/3T3 transformed by Kirsten MSV. J. of General Virology, 25: 207-218, 1974.

Pincus, T., Hartley, J.W., and Rowe, W.P.: A major genetic locus affecting resistance to infection with murine leukemia viruses. Virology, 1975. In press.

Pitha, P.M., Pitha, J., and Rowe, W.P.: Lack of requirement of reverse transcriptase function for the activation of murine leukemia virus. Virology 63: 568-572, 1975.

Sklar, M.D., White, B.J., and Rowe, W.P.: Initiation of oncogenic transformation of mouse lymphocytes *in vitro* by Abelson leukemia virus. Proc. Nat. Acad. Sci. 71: 4077-4081, 1974.

Project No. Z01 AI 00013-13 LVD
1. Viral Diseases
2. Viral Oncology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies of Viral Antigens in Virus-induced Tumors

Previous Serial Number: NIAID 71E

Principal Investigators: Drs. Andrew M. Lewis, Jr. and Wallace P. Rowe

Other Investigators: None

Cooperating Units: Drs. Arthur Levine, Cephas Patch, and James Breeden (Medicine Branch, NCI); Michael Oxman, Myron Levin (Harvard Medical School); Thomas J. Kelly, Jr., (Johns Hopkins University); Clyde S. Crumpacker, II (Beth Israel Hospital); Lenneart Philipson (University of Uppsala, Sweden); Satvir Tevethia (Tufts University); Sherman Weissman (Yale University).

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	31/12	12/12	0	43/12
Professional:	13/12	0	0	13/12
Other:	18/12	12/12	0	30/12

Project Description:

Objectives: To use the adenovirus-SV40 hybrid viruses as tools to study the genetics of DNA tumor viruses; to define the role of viral genes and antigens in the oncogenic process; to study the biology of adenovirus-SV40 hybrid viruses.

Methods Employed: Tissue culture, fluorescent antibody, complement fixation, serologic epidemiology, and nucleic acid homology, electron microscopic hetero-duplex mapping techniques.

Major Findings - Studies on nondefective adenovirus-SV40 hybrid viruses: The properties of the five nondefective adenovirus 2-SV40 hybrids which we have isolated and characterized were described in detail in the 1972-1973 project report. These hybrid viruses are genetic recombinants between adenovirus 2 and SV40 which contain between 6 and 48% of the SV40 genome inserted at the same location in the adenovirus 2 genome. Three of these hybrids

(Ad2^{+}ND$_{1}$, Ad2^{+}ND$_{2}$, and Ad2^{+}ND$_{4}$) induce SV40 U; U, TSTA; or U, TSTA, and T antigens respectively. The ability of these hybrids to induce more than 1 SV40-specific antigen is associated with the incorporation of increasingly larger segments of the SV40 genome into adenovirus 2 DNA. Thus, Ad2^{+}ND$_{1}$ which induces U antigen contains 17% of the SV40 genome while Ad2^{+}ND$_{4}$ which induces SV40 U, TSTA and T antigens contains 48% of the SV40 genome. By heteroduplex mapping techniques the regions responsible for inducing these SV40 functions have been located on the SV40 genome. In addition, using both nucleic acid hybridization and heteroduplex mapping, the SV40 DNA segments in each of the five nondefective hybrids have been mapped on the SV40 genome with respect to the cleavage sites of SV40 DNA produced by restriction endonucleases from Hemophilus influenzae and Hemophilus parainfluenzae.

1. Comparative efficiency of transformation of hamster and rat cells by Ad2 and the nondefective hybrids: During the past several years, we have developed a quantitative assay for evaluating the efficiency of transformation by Ad2. This assay consists of infecting cells with virus inactivated by exposure to ultraviolet light, plating out infected cells in plastic dishes and holding the cultures for a 3 month period before counting transformed foci. Studies in hamster embryo cells indicate that the efficiency of transformation by Ad2 is low requiring between $10^{6.7}$ plaque forming units of virus per focus forming units (PFU/FFU) to $10^{8.7}$ PFU/FFU depending upon the lot of hamster cells employed.

When the efficiency of transformation of the same lots of hamster cells by Ad2 and 3 of the nondefective hybrids (Ad2^{+}ND$_{2}$ containing 32% of SV40 genome, Ad2^{+}ND$_{4}$ and B55 each containing 48% of SV40 genome) was compared, we found that Ad2 and Ad2^{+}ND$_{2}$ transformed with similar efficiencies, $10^{7.6}$ and $10^{7.7}$ PFU/FFU respectively. Ad2^{+}ND$_{4}$ and B55 were slightly more efficient, transforming with efficiencies of $10^{7.2}$ and $10^{6.5}$ PFU/FFU respectively.

To support these results the experiments were repeated in rat embryo cells. In the same lot of rat cells two strains of Ad2 transformed at $10^{6.3}$ to $10^{7.1}$ PFU/FFU; while the nondefective hybrids, Ad2^{+}ND$_{2}$, Ad2^{+}ND$_{4}$ and B55, were more efficient requiring $10^{5.3}$, $10^{5.6}$ and $10^{4.7}$ PFU/FFU respectively. These data suggest that, in in vitro transformation assays, the efficiency of transformation by Ad2 varies with the lot and species of cells employed in the assay. The largest segment of SV40 DNA in the Ad2^{+}ND$_{4}$ and B55 hybrids appeared to increase the efficiency of transformation by hybrid virus compared with nonhybrid Ad2. Each of the hybrids tested was more efficient than Ad2 in transforming rat embryo cells. To confirm and strengthen these results, studies are underway to determine the particle infectivity ratio in the Ad2 and B55 virus pools and to compare by transfection the efficiency of transformation by Ad2 DNA and hybrid virus DNA.

2. Induction of tumors in hamsters with Ad2 transformed cell lines: To be certain that the foci being scored in the transformation assays represent colonies of cells with malignant potential a number of cell lines have been established and injected into newborn hamsters. Six lines of Ad2 transformed

hamster embryo cells have been studied thus far. One line Ad2HE$_1$ produced tumors in newborn hamsters that were readily transplanted to 21 day weanling animals. Titration of this cell line in 21 day old animals indicates that each cc of packed tumor cells represent between 10^3 to 10^4 tumor producing doses. A second cell line Ad2HE$_2$ has failed to produce tumors in newborn hamsters in several different tests even though as many as 10^7 cells have been injected into each newborn. Four other lines Ad2HE$_3$ through Ad2HE$_6$ produce tumors quite readily in newborn hamsters. When transplanted subcutaneously to hamsters 10 days old or older small tumors develop but usually regress over a 4 to 6 week period. The regression phenomenon is observed in 21 day old animals even after 10 to 12 passages of the tumor line in newborn animals. If the progressively enlarging tumors that develop in 21 day old animals are transplanted to other 21 day old animals the majority of animals develop small tumors that also regress. In addition 21 day old animals given massive inocula of tumor cells (25 times the dose of cells usually employed) usually clear these nodules within a 2 month period. Again when progressively enlarging tumors are removed from the occasional animal that develops such a lesion and transplanted to other 21 day old animals most develop small tumors that regress. Consequently, all attempts to adapt these tumors to hamsters over 10 days old have failed. We conclude at this time that maturation of the immune system is responsible for the rejection of these tumors in hamsters. Several experimental approaches are being evaluated to attempt to explain this regression phenomenon.

3. Heteroduplex mapping studies on the B55 hybrid: The B55 hybrid pool is the virus stock from which all 5 nondefective Ad2-SV40 hybrids were isolated. The hybrids containing the smaller SV40 segments were obtained by plaquing purifying the B55 hybrid in human kidney cells. When this virus was plaque purified in monkey kidney cells, however, the Ad2$^+$ND$_4$ hybrid was obtained. This hybrid contained 48% of the SV40 genome and was biologically indistinguishable from the B55 hybrid in that both induced all 3 early SV40 antigens (U, TSTA, and T antigens). It was of interest to examine the genome of the B55 hybrid and compare its DNA with Ad2$^+$ND$_4$ DNA by electron microscopic heteroduplex mapping techniques. By reacting B55 DNA with Ad2 DNA two types of DNA molecules were detected with approximately equal frequency. One type of duplex molecule contained a small substitution loop; the other type contained a much larger substitution loop. By reacting B55 DNA with itself and with Ad2$^+$ND$_4$ DNA we determined that the first type consisted of a Ad2-SV40 hybrid molecule containing 6.8% deletion of Ad2 DNA accompanied by a 10% insertion of the SV40 segment lying between 0.11 and 0.21 units on the SV40 map. Consequently, this hybrid was an intermediate between Ad2$^+$ND$_3$ (containing 7% of the SV40 genome and a 5.4% Ad2 deletion) and Ad2$^+$ND$_1$ (containing 17% of the SV40 genome and a 5.3% Ad2 deletion). The second type of Ad2-SV40 hybrid molecule was indistinguishable from the Ad2$^+$ND$_4$ hybrid in that it contained 48% of the SV40 genome associated with a 4.5% deletion of Ad2 DNA. No internal deletion such as Ad2$^+$ND$_4$ del were observed in the B55 hybrid molecule.

4. Induction of tumors in newborn hamster with U.V. inactivated Ad2$^+$ND$_4$ and B55: Several years ago we determined that none of the nondefective hybrids produced tumors when injected into newborn hamsters. Having demonstrated

that both infectious and ultraviolet light inactivated Ad2 will transform hamster cell in tissue culture and that these cells will induce tumors in hamsters, it was of interest to determine whether ultraviolet-light-inactivated Ad2 and the nondefective hybrids would induce tumors when injected into newborn hamsters. Consequently 2 strains of nonhybrid Ad2 and the nondefective hybrids, $Ad2^+ND_2$, $Ad2^+ND_4$ and B55 were exposed to 12,600 Ergs of ultraviolet light and injected into 4 litters of newborn hamsters. After 300 days of observation tumors have been detected in about 10% of the surviving animals injected with $Ad2^+ND_4$ or the B55 hybrid. No tumors have developed in animals injected with either strain of Ad2 or with $Ad2^+ND_2$. Animals injected with $Ad2^+ND_4$ which developed tumors also developed both Ad2 T and SV40 T antibodies in their serum. The tumor bearing animal infected with the B55 hybrid is currently under study. These studies are being repeated for confirmation.

Significance to Biomedical Research and Proposed Course of the Project:

Although our studies are incomplete and several essential experiments must be completed before firm conclusions can be made, it appears from the available data that the acquisition of that portion SV40 genome lying between the end of the SV40 segment in $Ad2^+ND_2$ (at .32 SV40 units) and the end of the ND_4/B55 segment (at .48 SV40 units) enhances the transforming ability of Ad2 in vitro and conveys upon this virus the ability to produce tumors when injected into newborn hamsters. This is the region of the SV40 genome which is associated with the induction of SV40 T antigen. Hopefully studies already underway will confirm these observations.

The consistent pattern of regression in weanlings hamsters of tumors induced in newborn hamsters by certain Ad2 transformed lines offers a unique model to study the immunological events resulting in spontaneous tumor regression. A low incidence of tumor regression has been observed in chickens with Rous sarcomas, in certain strains of mice carrying murine sarcomas and in a few children with neuroblastoma, however, the phenomenon generally appears too infrequently for careful study. To obtain regression consistently in greater than 90% of tumor bearing animals should provide significant advantage for detailed study of this phenomenon. Furthermore, any system in which a high incidence of spontaneous regression of malignant disease occurs should be carefully evaluated for mechanisms with potential application to control of human disease.

In addition to providing a useful model for study, the interaction between Ad2 virus transformed cells and their hosts which results in tumor regression indicates that host response is a vital aspect of the process of adenoviruses induced malignancy. It further suggests that during the process of transformation by adenovirus the transformed cell must either fail to induce enough substances which would confer immunity upon a susceptible host or must produce one or more parameters which allows them to overcome host resistance and establish a malignant process.

As mentioned above, studies are underway to confirm the in vitro and in vivo tumorgenicity studies. In addition, we are attempting to compare the transforming efficiency of hybrid and nonhybrid Ad2 DNA before and after digestion with E. coli R_1 endonuclease. These studies are being carried out in a collaborative containment unit in Building 41 provided on a short term basis (3-5 months) by NCI. DNA obtained from these agents will be supplied to Dr. Alex Van der Eb, Leiden, Netherlands who is collaborating in this project.

Given the association of a specific region of the SV40 genome with the process of tumor induction we must now consider isolating Ad2-SV40 hybrids (such as the $Ad2^{+}ND_4$ del) with deletions in this region and determine the effect of these deletions on T antigen induction and tumorgenesis.

In addition, the Ad2 transformed hamster cells must be carefully tested for the presence of Ad2 transplantation antigen to determine the role of this antigen in the rejection of certain lines of Ad2 transformed hamster cells.

In the last annual report I discussed at some length the impact of the lack of adequate containment facilities in NIAID on work with nondefective hybrids. Thirty months - 2 1/2 years - after the submission of my memorandum outlining the potential hazards attendant with work on nondefective recombinants between Ad2 and SV40 and 27 months after I discontinued work with viable nondefective hybrids there remains an absence of containment facilities in NIAID. Furthermore, there is no discussion of which I am aware of obtaining containment facilities to pursue such work within this Institute. A continuation of the studies on the oncogenicity of the nondefective Ad2-SV40 hybrids and the elucidation of the mechanism by which certain of these viruses induce tumors in hamsters will require the long term use of a containment facility designed for moderate risk viruses. If NIAID is to participate in this area of research the development of such facilities is essential.

Key Descriptors: Viral-antigen, virus-induced tumors, adenovirus-SV40 hybrid viruses, recombinants, transformation, transformation efficiency, tumor regression, genetic mapping.

Publications:

Lewis, A.M., Jr., Rabson, A.S., and Levine, A.S.: Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. X. Transformation of hamster kidney cells by adenovirus 2 and the nondefective hybrid viruses. J. Virol., 13: 1291-1301, 1974.

Kelly, T.J., Jr., Lewis, A.M., Jr., Levine, A.S., and Siegal, S.: Structure of two Ad2-SV40 hybrids which contain the entire SV40 genome. J. Mol. Biol. 89: 113-126, 1974.

Lewis, A.M., Jr., Breeden, J.H., Wewerka, Y.L., Schnipper, L.E., and Levine, A.S.: Studies of hamster cells transformed by adenovirus 2 and the nondefective adenovirus 2-SV40 hybrids. In Cold Spring Harbor Tumor Virus Symposium, 39: 651-656, 1974.

Siegel, S.E., Patch, C.T., Lewis, A.M., Jr., Levine, A.S.: SV40 DNA replication, transcription and antigen induction during infection with two adenovirus 2-SV40 hybrids which contain the entire SV40 genome. J. Virol., in press

Project No. Z01AI00014-09
1. Laboratory of Viral Diseases
2. Molecular Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on Interferon and Interferon Inducers

Previous Serial Number: NIAID-71F

Principal Investigator: Dr. Hilton B. Levy

Other Investigators: Mr. Freddie Riley
Dr. Eduard Lvovsky

Cooperating Units: None

Man Years:

Total:	22/12
Professional:	5/12
Other:	17/12

Project Description:

Objectives

To analyze the molecular alterations in interferon treated cells that may explain their resistance to virus infection.

Methods Employed

A wide variety of biochemical, biophysical and virological techniques have been employed.

Major Findings

This laboratory, along with others, showed several years ago that interferon-treated cells have the ability to translate normal cell mRNAs while being strongly restricted in their ability to translate viral mRNAs. It was also shown that one of the alterations induced by interferon was a modification of ribosomes or ribosome-associated factors. However, the mechanism by which "interferon type" ribosomes are able to distinguish between viral and cellular mRNAs is not understood. We recently reported interferon-induced alterations in RNA species associated with polysomes in noninfected cells. These alterations in RNA species appear to be associated with the interferon-induced antiviral activity. Reported here are data which relate to the type of RNA that are altered and the nature of these alterations.

The data gathered during the past year show that these RNA species are poly (A) containing mRNAs without a poly (A)-rich region and tRNAs. In addition, it was shown that in polyacrylamide gels in aqueous medium as well as in non-aqueous medium(formamide) the mRNAs from interferon treated cells migrated more slowly than do control cell mRNAs, suggesting that the interferon mRNAs are slightly larger than normal. Transfer RNAs from interferon treated cells on the other hand, move more slowly in aqueous medium, but not in formamide, suggesting that the difference in mobility in tRNAs is associated with factors other than size, possibly secondary structures.

A publication on this work is being prepared for publication in P.N.A.S.

Keyword Descriptors: Interferon, mechanism of action, action of interferon on uninfected cells.

Publications:

Kohno, S., Buckler, C. E., Levy, H. B., and Baron, S.: Elution of cell-bound interferon as a cause of development of antiviral activity subsequent to washing of interferon-treated cell cultures. IRCS Med. Sci: Cell Memb. Biol., Immunol. Allergy, Microbiol., Parasit., & Inf. Dis. 3: 57, 1975.

Levy, H. B., and Riley, F. L.: Interferon induced alterations in cell macromolecules. In Report of an interferon workshop. Gulbenkian Foundation Lisbon, Portugal, 1975.

Levy, H. B., Buckler, C. E., and Riley, F.: Interferon. in Nayak, D., (ed): Biochemistry of Virus. New York, Marcel Dekker, 1975, in press.

Project No. Z01AI00015-09
1. Laboratory of Viral Diseases
2. Cellular Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30 1975

Project Title: Structure, Properties and Replication of Viruses of Pathogenic Protozoa and Mammals.

Previous Serial Number: NIAID-71-G

Principal Investigator: Dr. C. F. T. Mattern

Other Investigators: Wendell A. Daniel, M. S.
Anna M. Kontonis, M. S.

Cooperating Units: Dr. Louis S. Diamond, LPD, NIAID
Dr. James A. Rose, LBV, NIAID
Dr. Malcolm Martin, LBV, NIAID
Dr. Shigeko Nomura, VLL, NCI

Man Years:

Total	24/12
Professional	6/12
Other	18/12

Project Description:

Objectives

The study of protozoal viruses; morphogenesis and architecture of mammalian viruses.

A. Ameba Viruses: In collaboration with Dr. Louis S. Diamond, we are placing a major emphasis on understanding viruses of Entamoeba histolytica, especially regarding the carrier vs. lysogenic state, their possible role in pathogenicity, and purification of the two or more virus forms. The filamentous virus appears to band in CsCl at a density of about 1.29 and morphologically it resembles the 7 nm diameter filamentous bacteriophages. The icosahedral viruses appear to band at about 1.33-1.34 density, but are completely unstable upon dialysis. Efforts to stabilize them are in progress.

A novel beaded "virus" has been observed by infecting susceptible amebal strains with viruses of three different donors. This new agent, possibly a third amebal virus, is under continued study.

A fourth bacteriophage-like entity from Entamoeba has been observed and is being studied.

We have been successful in inducing a susceptible amebal strain to become "lysogenic" for a heterologous icosahedral virus. It continuously produces low levels of the "new" virus, as determined by host range, and it is resistant to superinfection. This model system is being investigated to see if amebal virulence in hamster liver is related to the amebal virus type.

Preliminary studies suggest that V_{458}, which virus has the broadest host range of any amebal virus, might consist of more than one virus type. Segregation of potential components is being pursued by limiting dilution passage in various amebal hosts followed by periodic testing of the progeny host range.

Forty-three clones of HB-301 amebae, representing 3 clonal generations have been established following prolonged antiserum treatment. All clones have been shown to produce virus.

Clones of HM-1 (=ABRM) amebae have also been established and all clones tested produce virus. These clones have been shown to produce both filamentous and icosahedral viruses indicating they are either double carriers or doubly lysogenic.

To date all attempts to infect animals or a wide variety of mammalian cells in tissue culture have been unsuccessful. Likewise we have been unable to find a virus-free amebal strain or to cure an amebal culture of its virus.

Keyword Descriptors: Viruses, Entamoeba histolytica, amebal amebal viruses; ultrastructure.

Publications:

Nomura, S, Dunn, K. J., Mattern, C. F. T., Hartley, J. W. and Fischinger, P. J. Revertants of mouse cells transformed by murine sarcoma virus:flat variants without a rescuable sarcoma virus from a clone of BALB/3T3 transformed by Kirsten MSV. J. Gen. Virol. 25: 207-218, 1974.

Papageorge, A. G., Peebles, P. T., Gerwin, B. I., Fischinger, P. J. and Mattern, C. F. T. Expression of the defective "S+L-" type murine sarcoma virus genome in human amnion and lung cells. J. Natl. Cancer Inst. 52: 1727-1737, 1974.

Diamond, L. S. and Mattern, C. F. T. Animal Virus. in McGraw Hill yearbook of Science and Technology. in press 1975.

Diamond, L. S. and Mattern, C. F. T. Viruses of *Entamoeba histolytica*. in Advances in Virus Research., in press, 1975.

Project No. NIAID Z01AI00016-09
1. Laboratory of Viral Diseases
2. Cellular Virology Section
3. Bethesda Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biological Ultrastructure

Previous Serial Number: NIAID-71-G1

Principal Investigator: Dr. Carl F. T. Mattern

Other Investigators: Wendell A. Daniel, M.S.
Nava Paran, M.S.

Cooperating Units: Dr. Bronislaw N. Honigberg, Department of Zoology, Univ. of Mass. Amherst
Dr. Ruth Pertel, LPD

Man Years:

Total:	18/12
Professional:	12/12
Other:	6/12

Project Description:

This project, which has been in existence for about 14 years is being largely phased out. In the past we have investigated the ultrastructure of protozoa, metazoa and vertebrate organelles emphasizing phylogenetic kinships and embryogenesis.

The past year has been devoted largely to writing up details of completed experiments and there are therefore no additional findings over and above those reported in prior years. Two papers have been published, four additional papers submitted, and two further papers are to be prepared.

Certain aspects of this project will be incorporated into Virus Project NIAID 71-G in that future ultrastructural studies on pathogenic protozoa will involve their relationship to mammalian cell systems, including searching for indigenous viruses, toxins and the study of their pathogenicity.

Keyword Descriptors:

A. Trichomonas vaginalis, ultrastructure, division forms.
B. Mammalian taste buds, ultrastructure, histochemistry, acetylcholinesterase, contractile mechanism.
C. Caenorhabditis elegans, amphids, histochemistry acetylcholinesterase, cephalic papillae.

Publications:

Mattern, C. F. T. and Paran, N.: Evidence of a contractile mechanism in the taste bud of the mouse fungiform papilla. Exp. Neurol. 44, 461-469, 1974.

Paran, N. and Mattern, C. F. T.: The distribution of acetylcholinesterase in buds of the rat vallate papilla as determined by electron microscope histochemistry. J. Comp. Neurol. 159: 29-44, 1975.

Pertel, R., Paran, N. and Mattern, c. F. T.: Ultrastructural localization of cholinesterase associated with the anterior structures of Caenorhabditis elegans. Exp. Parasitol, in press, 1975.

Paran, N., Mattern, C. F. T., and Henkin, R. I.: Ultrastructure of the taste bud of the human fungiform papilla. Cell and Tissue Res., in press, 1975.

Mattern, C. F. T., and Paran, N.: Observations on the origin of the dense extracellular pore substance of the bovine taste bud. Trans. Amer. Microsc. Soc., in press, 1975.

Project No. Z01AI00017-09
1. Laboratory of Viral Diseases
2. Cellular Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Host Defenses Against Viral Infection: Interferon and Immunity

Previous Serial Number: NIAID-71-H

Principal Investigator: Dr. Samuel Baron

Other Investigators: Dr. Jon Green
Dr. F. Dianzani

Cooperating Units: Dr. H. M. Johnson, FDA, Cincinnati, Ohio
Dr. Ira Green, LM, NIAID
Dr. George Baer, Center for Disease Control, Atlanta Ga.
Dr. S. Kohno, NIH, Tokyo, Japan
Dr. G. D. Gordon, NIDR

Man Years:

Total:	43/12
Professional:	29/12
Others:	24/12

Contract Support:

Contract Number	Contract Principal Investigator	Objectives and Findings	FY75,FY76 Funding Plans
73-C-854CC	North American Biologicals Dr. R. Rafajko	Service contract to perform interferon and inducer assays for research below.	$51,514
75-C-462CC	Biofluids Dr. R. Rafajko	Service contract to perform interferon and inducer assays for research below.	$59,108

Project Description:

Objectives

To continue to elucidate mechanisms which control host defenses (interferon and immunity) during viral infections of the whole animal and of cultured cells so that methods might be developed to employ these mechanisms to control human viral infections.

Methods Employed

The interferon system and the immune system were studied in vitro using cell cultures from different animal species including man. In vivo studies of host defenses during viral infection were carried out in mice, rabbits and sub-human primates. The viruses used included herpes simplex virus, vesicular stomatitis virus, Sindbis virus, Semliki Forest virus, CF-7 virus, and Simian hemorrhagic fever virus. The materials employed included interferons from various species, antibody, mouse spleen cells, interferon inducers and metabolic inhibitors.

Major Findings

Interaction between the interferon system and the immune system. The cell population necessary for interferon production after mitogen stimulation was investigated in the mouse. Thymus cells and cortisone-resistant thymus cells failed to produce interferon after mitogen stimulation. When spleen cells, a mixture of B and T cells, were treated with anti-K chain serum and complement and then stimulated with PHA, no diminution of interferon production was observed. However, when spleen cells were treated with antitheta antibody and complement and then stimulated with PHA, interferon production was markedly reduced. These studies suggest that peripheralized T cells are required for interferon production after mitogen stimulation.

To help determine the natural role of the lymphocyte produced interferon during antibody induction and production, we have investigated the effect of several interferon preparations on the primary in vitro antibody response. The primary in vitro plaque forming cell response of mouse spleen cells to sheep red blood cells was inhibited by two sources of crude mouse interferon and two sources of highly purified interferon. The substances in the interferon preparations which induce the pfc and antiviral inhibitory activity were found to be the same by their common properties. The inhibitory effect of interferon appears to affect an early event in the antibody forming system. Preliminary data on the primary in vitro pfc response to a T cell independent antigen (O antigen of E. coli) demonstrated that interferon is inhibitory for B cell function as well as T cell function. These findings raise the possibility that during the sensitization of an animal to an antigen the antibody response can be limited when sensitized T cells react with antigen and in the resulting mitogenic response produce interferon as one of the lymphokines. The interferon so produced is in sufficient concentration to regulate subsequent antibody induction or production.

Reaction of interferon and cells. It had previously been suggested that binding of approximately 1% of applied interferon to cell surfaces is the initial step leading to antiviral activity. This conclusion was based largely on the finding that antiviral activity develops after washing away unbound interferon and the antiviral activity was correlated with the presence on the cell surface of a small fraction of the applied interferon. An alternative explanation is that the cell bound interferon served as a reservoir which after washing replenishes extracellular interferon at a low concentration. In this way the concentration of eluted interferon in the medium would govern the development of the antiviral state by reaction with the cells. The alternative explanation was supported by the findings that (1) cell bound interferon eluted into the medium by 30 minutes of incubation but antiviral activity developed subsequent to the elution and (2) in experiments where the elution volume was varied, the level of viral resistance was inversely proportional to the elution volume indicating that the eluted interferon concentration determined the level of interferon induced resistance. It is reasonable to believe that some part of the detectable, bound interferon is a specific first step for induction of resistance but the specific fraction of the total bound interferon and the required time of contact with the cell remain to be defined.

Late interferon therapy of viral infected cells. Late therapy of certain viral infections of animals has become possible as increased amounts of interferon and more effective inducers have become available. It has not been determined whether the protective effect of this interferon is on cells already infected or whether interferon protects cells not yet infected. Therefore, a study in mouse L cell cultures was undertaken to help determine the viral inhibitory effect of 3,000 units/ml of interferon applied at different times during single cycle multiplication. Interferon lost its effectiveness when applied 1 to 3 hours after initiation of infection. In comparison an inhibitor of protein synthesis (cycloheximide) inhibited the yield of virus throughout the 10 hours of the virus growth cycle. These findings imply that in vivo interferon may act to protect cells recently infected and also cells not yet infected with these viruses but not cells infected more than several hours.

Protection of monkeys against Simian hemorrhagic fever. The nuclease resistant poly I.poly C-poly-l-lysine-carboxymethylcellulose interferon inducing complex developed by Levy et al. was determined in collaborative studies with Drs. Levy and London to prophylactically protect monkeys against Simian hemorrhagic fever. Preceeding studies had indicated that the virus in tissue culture was sensitive to the antiviral action of interferon. This is the first reported control of a systemic virus disease in a non-human primate by an interferon inducer. Dr. Levy's report provides additional information.

Studies of the relationship of recurrent herpes simplex virus in man to virus growth and interferon activity in human gingival cultures. Cultures of human gingival fibroblast cells from patients with a positive or negative history of recurrent herpes simplex virus infection were compared with respect to susceptibility to herpes simplex virus infection and interferon activity. No correlation was found between prior infection and interferon production or susceptibility of the cells to herpes simplex virus infection. The numbers were too small to rule out a possible inverse relationship between interferon sensitivity of gingival fibroblast cells and a clinical history of recurrent disease.

Significance to Biomedical Research and Proposed Course of the Project

Studies of the possible immune-regulatory role of interferon during the immune response helped to further define the complex natural mechanism of immune regulation and the role of lymphokine-interferon in that process. The findings suggest the possible usefulness of interferon as an immune suppressant which potentially could be used during organ transplantation. Interferon would have the advantage of being a natural product with presumably low toxicity. Studies will be continued to further define the role of interferon during the various stages of the immune response

The continued development of knowledge of the interaction of interferon with the cell surface leading to the antiviral state will provide the basis for more rational development of interferon induced antiviral activity during viral infections.

The finding that interferon treatment of acutely infected cells lost efficacy 1 to 3 hours after infection indicates possible limits to treatment of already infected cells and suggests that therapy of infected individuals be directed at protecting cells not yet infected. Further study of late therapy of virus infected cells with interferon must await development of more potent and more pure preparations of interferon.

The efficacy of interferon induction in sub-human primates for protection against viral infection confirms and extends the protection previously observed in other animal species. Considering the closeness of monkeys to man, the finding tends to confirm that effective induction of interferon in man may be similarly effective. Studies of prophylaxis and treatment of viral infections in primates will continue to help define the conditions needed for control of similar infections in man.

Correlations of sensitivity of cultured gingival cells from patients with and without recurrent herpes simplex virus infection have tended to rule out possible genetic differences in the cells of patients with recurrent disease as compared to patients without recurrent disease with regard to sensitivity to virus infection and interferon activity. The study will be continued to obtain more patients to rule in or rule out the possibility that sensitivity of the cells to interferon may be a factor in the susceptibility to recurrance.

Keyword descriptors: viruses, host defenses, interferon, antibody, cell mediated immunity.

Publications:

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Project No. Z01AI00018-09
1. Laboratory of Viral Diseases
2. Cellular Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biological and Biochemical Characterization of Human Papovaviruses

Previous Serial No.: NIAID-71J

Principal Investigator: Dr. Kenneth K. Takemoto

Other Investigators: Dr. Michael F. Mullarkey, LVD
Dr. David Mason, LVD

Cooperating Units: Dr. Malcolm A. Martin, LBV, NIAID
Dr. Peter Howley, LBV, NIAID
Dr. George Khoury, LBV, NIAID
Dr. Alan Rabson, NCI

Man Years:

Total: 5
Professional: 3
Others: 2

Project Description:

Objectives

1. To characterize as completely as possible, 2 newly discovered papovaviruses of humans, the JC and BK prototype viruses, as well as 3 other new isolates made in this laboratory. Biological studies include: (1) Host range for optimal growth of these viruses, (2) oncogenic and transforming activities, (3) antigenic relationship to each other as well as to the simian virus 40, (4) possible role in disease. Biochemical studies done in collaboration with Dr. Martin's group include: (1) relatedness of the DNA's of the different viruses, (2) biochemical and physical maps of the DNA from the various isolates. (3) Transformation with viral DNA and fragments derived by restriction endonuclease cleavage.

Methods Employed

Standard virological and serological methods as well as cell culture techniques are employed.

Major Findings

As in the past year, our major efforts have been in characterization of the 2 new human papovaviruses (the BK and JC prototypes). In addition, 3 new isolates were made in this laboratory from Wiskott-Aldrich patients. These new isolates are very closely related to the BK virus. Of special interest and possible importance is the continuous excretion of virus in the urine of these patients. Two of the patients developed malignant diseases, and virus was isolated from the tumor of one of them; the tumor from the other could not be studied due to massive fungal infection.

Characterization of human papovaviruses, BK and JC: 1. Replication. The BK related viruses grow well in a variety of human cells and can be quantitated by the plaque assay in human embryonic kidney cells. In monkey cells, the infection is predominantly abortive, and only a few cells produce infectious virus. The JC virus, which has been associated with progressive multifocal leukoencephalopathy (14 cases thus far) is still a difficult virus to investigate because of its restricted host range. We have been able to propagate the virus in large quantities, but only in human fetal brain cells. Due to difficulties in obtaining fetal brains, progress has been hampered by lack of suitable cultures. However, a sufficiently large stock has now been grown to proceed with studies on this virus. Attempts to cultivate the JC virus in human embryonic kidney as well as different strains of fibroblasts have been unsuccessful.

2. Infection of animals: Hamsters inoculated with BK as well as 1 of the Wiskott-Aldrich isolates did not develop tumors during 1 year of observation, nor did any disease develop. Rabbits, like hamsters, did not develop any symptoms of disease although they produce high antibody titers against viral antigens. Like humans, hamsters and rabbits do not develop detectable antibody to the non-virion T-antigens. On the other hand, 5 newborn rhesus monkeys which had been inoculated at birth (intracerebrally, intraperitoneally, and subcutaneously) developed high antiviral and anti-T antibodies over a period of one year. These animals will be observed for another 2 years for possible diseases.

3. Transformation. Unlike SV40, BKV is highly inefficient in transforming cells in culture. Thus far, BKV has not transformed hamster, mouse, and rat cells in a number of different experiments. On the other hand, viral DNA rapidly transformed hamster cells, and transformed colonies appeared within 3 to 4 weeks. The cells contain T-antigens which react with SV40 and JC T-antibodies. Tumors were produced when cells were inoculated into young hamsters. The serum from tumor-bearing animals contained T-antibody which reacted with its own as well as SV40 transformed cells. In addition, an apparently unique antibody was detected in BK hamster tumor serum which reacted with normal cells (anti- nuclear antibody).

The anti-nuclear antibody (ANA) reacted with normal cells from a variety of cells from different species; human, mouse, hamster, rat, monkey, and chick cells show patterns of fluorescence indistinguishable from that seen in T-antigen staining. The tumor is now being carried in serial transplant to determine whether transplanted tumors also give rise to an antibody response against normal cellular antigens.

Comparison of SV40 functions with BK. Previous studies from this laboratory have shown that BK and SV40 share some of the early viral functions, specifically the induction of a common T-antigen. Other early functions of SV40 have now been compared with BK and these results can be summarized as follows: (1) BK induces the SV40 "U" antigen as demonstrated by reaction of BK infected cells with anti-U serum supplied by Dr. A. Lewis. Furthermore, BK T-antibody stains HEK cells infected with the ND-1 Adeno-SV40 hybrid which induced "U" antigen only. (2) Preliminary evidence suggests that BK will complement the "early" temperature sensitive mutants of SV40 (Group A mutants). These mutants cannot replicate at high temperature due to defects in early functions; the block can be overcome by coinfection with BKV which apparently provides the missing functions. (3) Attempts to demonstrate "helper" activity for adenovirus growth in African green monkey cells have been unsuccessful, but these experiments are still inconclusive.

Growth of JC virus. Most laboratories attempting to grow JC virus have failed due to its highly restricted host range. Because of the importance of this virus in PML disease of humans, major efforts have been made to determine optimal methods for growth of this virus as well as exploration of other more easily attainable host cells. The virus has been successfully propagated only in human fetal brain cells; virus growth is extremely slow, and takes approximately 1 month for maximum yields. However, a sufficient amount of isotopically-labelled virus was prepared to do comparative studies with SV40 and BKV. These studies, conducted in collaboration with Drs. Howley and Martin of LBV, have shown that there is only a 20% homology between the DNA's of JC and BK, and 10% between JC and SV40. Like BK, the JC virus shows cross reacting virion (V) and tumor (T) antigens with SV40 Thus all three viruses, BK, JC, and SV40 show relatedness of their V and T antigens.

Comparative studies of T-antigens of BK, JC, and SV40. By a combination of serologic techniques the T-antigens of BK, JC, and SV40 have been examined for possible differences. By these techniques, no differences have been detected thus far. Further experiments involving the purification of the T-antigens are in progress.

Significance to Biomedical Research

The human papovaviruses are widespread in the human population, and serologic evidence for infection by these viruses has been found throughout the world. These are recently recognized viruses of humans and their importance in disease requires investigation. Because of their persistence after infection and their finding in human brains in association with the demyelinating disease, progressive multifocal leukoencephalopathy, they may be important pathogens in human disease. Furthermore, because of their oncogenicity for lower animals and their ability to transform cells in culture, their role in malignant diseases of humans needs to be determined.

Proposed Course of the Project

Investigations on the characterization of human papovaviruses will be continued and extended. With the accumulation of large stocks of JC virus, work will be expanded on this virus which is still poorly understood. Major efforts will be made to adapt the virus to growth in other cells. Since several laboratories (including this laboratory) have reported either isolation or identification of papovaviruses in human tumors, efforts will be made to study these viruses in human malignancies.

Keyword Descriptors: 1. Human Papovaviruses; 2. Oncogenic Viruses; 3. Slow Viruses; 4. Progressive Multifocal Leukoencephalopathy

Publications:

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Project No. Z01AI00019-01
1. Laboratory of Viral Diseases
2. Molecular Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the Treatment of Disease with the Interferon System

Previous Serial Number: None

Principal Investigator: Dr. Hilton B. Levy

Other Investigators: Charles Buckler, LVD, NIAID

Cooperating Units: Dr. George Baer, CDC, Atlanta, Ga.
Dr. Edward Stephen, Ft. Detrick, Md.
Dr. Gerald Eddy, Ft. Detrick, Md.
Dr. William London, NINDS
Dr. Clarence Gibbs, NINDS
Dr. Herbert Kaufman, Department of Ophthalmology, Univ. Florida
Dr. Christian Anfinsen, NIAMDD

Man Years:

Total:	12/12
Professional:	5/12
Other:	7/12

Project Description:

Objectives

To develop and test an interferon inducer that is effective in primates, including man. To develop methods for interferon production and analysis that will lead to structure determination and synthesis.

Major Findings

Poly I.poly C is a good interferon inducer, a good antiviral agent and a good antitumor agent in rodents, but is ineffective in man. In monkeys and chimpanzees it was even less effective than in man, inducing no interferon in the non-human primates. Last year we reported the preparation of a stabilized poly I.poly C derivative, containing poly-l-lysine and carboxymethylcellulose. We have studied this material further this past year, with the following conclusions; the complex is much more stable than poly I.poly C. It has a thermal denaturation temperature about 40C higher than poly I.poly C. Its resistance to hydrolysis by ribonuclease or primate serum is 10-15 X that of poly I.poly C. Fairly extensive toxicology studies indicate that the complex is no more toxic and perhaps less toxic in large mammals, including primates than is poly I.poly C.

The new drug has been used in attempts to control several virus diseases in monkeys. For the most part, but not exclusively, these studies have been prophylactic rather than therapeutic in design. Four virus diseases have been examined.

1) Poly I.poly C.poly-l-lysine (=PIC-L) completely protects monkeys against Simian hemorrhagic fever virus. This virus is uniformly fatal in untreated monkeys. PIC-L treatment completely aborted this infection, but there was no development of antibodies or immunity. No viremia developed in the treated animal and animals were completely susceptible to subsequent re-challenge.

2) Complete protection against LD_{100} challenges of yellow fever virus was obtained, even when the treatment was begun 8 hours post infection. With yellow fever virus, there was some (reduced) viremia. Very high levels of neutralizing antibodies and immunity to subsequent re-challenge were found.

3) Complete post exposure prophylaxis vs. street rabies virus in monkeys was obtained. Antibody data are being evaluated.

4) Prophylaxis vs. herpetic keratoconjunctivitis in owl monkeys has been obtained. Interferon is produced in the monkeys tears following ocular instillation of PIC-L. No visible toxicity was noted.

Studies of the various effects that interferon has on cells as well as studies of the biochemical action of interferon would be considerably enhanced by the availability of purified interferon. In order to support efforts to purify human interferon being conducted by Dr. C. Anfinsen in NIAMDD, methods for the production of large amounts of radio labeled human interferon need to be developed. Studies are underway to determine the optimal conditions necessary to produce ^3H labeled human interferon in cultures of human lymphoblastoid cells induced by Sendai virus. Maximum titers of crude human interferon produced so far are between 10,000 and 30,000 units/ml. The parameters that have been optimized include; pH of cell culture medium, aeration of cultures, amount of Sendai, the kinetics of interferon production, serum concentration in the induction culture, concentration of cells and need of preincubation of the cells with interferon (priming).

The best conditions that have been developed to date are non-primed cells at 1 X 10^7 cells/ml, 4% fetal bovine serum in medium RPMI 1640, 600 HA units Sendai virus/ml cells and control of medium pH between pH 6.5 to 7.2. The interferon is produced by 9-12 hours after Sendai induction. Further parameters such as inducers (other viruses or poly I.poly C) and culture vessel geometry are under investigation.

Additional studies are being conducted to determine if small molecular weight, active fragments of interferon can be obtained. Preliminary investigations suggest that gentle digestion of mouse interferon by trypsin may indeed produce an acitve fragment of about 18,000 molecular weight (Native mouse interferon mw. 25,000). These studies are being extended, and other methods of fragmentation and other interferons will be investigated.

Proposed Course of the Project

The very encouraging effects of PIC-L in monkeys justifys trials in man, for control of both viral diseases and malignancies. An IND has been approved by the Bureau of Biologics for trial in leukemia patients with Dr. Arthur Levine. With Dr. Chanock we plan to try the compound topically for respiratory viruses. With Dr. Herbert Kaufman we plan to test its effect in chronic herpes keratoconjunctivitis in man.

About a year ago, the B.O.B. said that if the interferon system can be shown to be efficacious in rabies in monkeys, they would consider recommending its inclusion in the standard treatment for rabies. We plan to explore this question with the B.O.B.

Keyword Descriptors: Interferon, interferon inducers, virus therapy, primates, simian hemorrhagic fever, yellow fever, rabies.

Publications:
Levy, H. B., Baer, G., Baron, S., Gibbs, C. F., Iadarola, M., London, W., and Rice, J. M.: Induction of interferon in non-human primates by a nuclease-resistant polyriboinosinic. polyribocytidylic acid complex. IRCS: Microbiol. Parasit. & Inf. Dis. 2: 1643, 1974.

Project No. Z01AI00020-09
1. Laboratory of Viral Diseases
2. Molecular Virology Section
3. Bethesda Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Study of the factors, in vivo and in vitro which modify the outcome of chronic virus infections, using Lactic Dehydrogenase Virus (LDV) and its host cell: the mouse macrophage as a model.

Previous Serial Number: NIAID-711

Principal Investigator: Dr. Herman G. duBuy

Other Investigators: None

Cooperating Units: Dr. Hilton Levy, LVD, NIAID
Dr. Samuel Baron, LVD, NIAID
Dr. Carl F. T. Mattern, LVD, NIAID
Dr. Eugene Weinbach, LPD, NIAID

Man Years:

Total	27/12
Professional	12/12
Other	15/12

Project Description:

Objectives

Study of the factors which might modify the outcome of a chronic LDV infection. These are: Interferon and its inducers; induced changes in humoral immunity and in cellular immunity, by inducing changes in the macrophages, the host cells for LDV.

Methods Employed

For the production of high titers of mouse interferon, needed for the study of its effect on chronic viruses: the in vivo and in vitro cultivation of a high-IF-producing cell line, to determine the conditions leading to maximum interferon production.

For determination of the effect of the factors which modify the outcome of chronic virus infections: titration of LDV concentrations in the plasma of treated mice, or the supernatants of treated macrophage cultures.

Major Findings

The study on the role of the interferon inducer In.Cn on murine LDV infection in vivo showed that prophylactic treatment as well as therapeutic treatment, either with one or multiple doses, led to only a temporary decrease in viremic titers. The results were comparable to those obtained with exogenous interferon administration, as published previously.

The study on the role of agents which change macrophage function led to the finding that silica injection induced proliferation of new macrophages, thus aggravating the chronic viremia.

The study on the maximal production of interferon showed that the C243 mouse cell line, previously adapted by me to ascitic growth in general purpose mice, and occasionally yielding 20 ml ascitic fluid/mouse, allowed in vitro cultivation of these cells for 12 hours, at a concentration of 10^7 to 10^8 cells per ml. When stimulated by NDV, a yield of more than 10^5u of interferon per ml was found. This amount of interferon, probably the highest concentration ever produced, will allow the use of high concentration of exogenous interferon to try to eliminate the chronic LDV viremia.

This study on the ascitic C243 is also of interest because this C243 ascites, in contradistinction with other ascitic tumors, e.g. Ehrlich ascites, (1) does not kill the mice in 4-6 weeks and (2) has a relatively high rate of "spontaneous" regression.

Thus it presents a valuable model for a number of human ascites tumors, which seem to act similarly, and for which no animal model was available.

Significance to Biomedical Research and the Program of the Institute; and Proposed Course

Since Dr. Baron and Dr. Levy are interested in methods of high interferon production, the results obtained might be of use to them.

The results with silica further elucidate the possible effect of air pollutants on chronic virus infections.

Application of the findings regarding the interferon production by C243 mouse cells to cell cultures producing human interferon, in collaboration with Dr. Levy.

Application of the techniques for LDV titering to the LDH-enzyme changes caused by infectious diseases other than viruses, in collaboration with Dr. Weinbach's unit, specifically with Dr. T. Mercado.

Use of purification techniques of subcellular elements and viruses for the purification of certain protozoal subcellular elements, in collaboration with Dr. Mattern's unit.

Keyword Descriptors: Lactic Dehydrogenase Virus. C243 mouse ascites. Silicon-macrophages.

Publications:

Johnson, H. L., duBuy, H. G.: Effect of a potent interferon inducer on acute and chronic lactic dehydrogenase virus viremia. Infection and Immunity. 11: 113-116, 1975.

duBuy, H. G.: Effect of silica on virus infections in mice and mouse tissue culture. Infect. Immun. 11: 996-1002, 1975.

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LBV

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LPD

LABORATORY OF STREPTOCOCCAL DISEASES

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PHS-NIH
Summary Statement
Laboratory of Streptococcal Diseases
July 1, 1974 through June 30, 1975

Research Progress

New Viruses Found in a Mycoplasma

Two years ago, when we described the ultrastructure of a new genus and species of plant mycoplasma (*Spiroplasma citri*) a long-tailed bacteriophage was found infecting the microorganism. This was the first instance of a phage infecting a true mycoplasma, although a short-tailed phage was later found, and two other types of virus particles were previously known, in another wall-less organism, *Acholeplasma laidlawii*. We have subsequently found, by electron microscopic examination, that a rod-shaped virus and a short-tailed phage also occur in *S. citri*--and that, in fact, all three viruses may occur simultaneously in the same strain. To date, all 27 strains, received from 5 different laboratories in 4 countries, contain one or more such viruses. No strains suitable as indicators, and therefore for propagation of virus in large numbers, have yet been found: the viruses are, therefore, yet uncharacterized by infectivity, host-range, nucleic acid type, and other attributes. Although seen sporadically by the necessary electron microscopic examination, an average of at least 74% of passages examined are virus-positive,and testing of various media resulted in selection of one which, in a short-term trial, reveals virus production in 89% of passages examined. We expect that these viruses will offer useful probes in a host system uncomplicated by cell walls, for ready study of such basic biological attributes as membrane structure, the nature and distribution of virus receptors, and various aspects of intracellular viral replication. In addition, they may be of taxonomic interest, because microorganisms which appear to be other spiroplasmas are now being cultured from different plants and some insects. (Cole, Mitchell, Popkin, Tully)

Streptococcus sanguis or "Group H" is not a Homogeneous Grouping

Streptococci designated as *Streptococcus sanguis* are now known to be commonest on tooth surfaces, in dental plaque, and as a cause of subacute bacterial endocarditis. Their precise identity with serologic "Group H" streptococci, which were described earlier, has been a matter of debate--and of interest to us because the latter includes the genetically transformable strains. Despite lack of information, a number of designated strains of both *S. sanguis* and "Group H" appear to be "classic", and have been used repeatedly for over 30 years as reference strains. To aid in clarification, we examined 28 such "classic" strains by patterns of several attributes, which included: sensitivity to a cell-wall splitting muralysin, antigenic composition by immunoelectrophoresis, reactivity with a "Group H" antiserum and a "non-H" antiserum, adsorption of a Group H bacteriophage, the presence of cell surface projections or fimbriae, and the presence and amounts of cell-wall sugars--as well as by standard physiological tests. All strains were *S. sanguis* by the latter, but serologically they fell into 4 distinct groupings, or types: this distribution was reflected by patterns of the other attributes. Three of the types were easily distinguishable by consistent patterns of muralysin sensitivity and simple serology; the fourth required use of patterns of additional attributes. The results clearly show that strains that are S. sanguis physiologically are not homogeneous serologically nor by other characteristics; and therefore suggest caution, both in the selection of reference strains for genetic or other purposes, and in attempting to determine the nature of the "H" antigen and the validity of an "H" serogroup. (Cole, Calandra, Huff; Facklam and Rosan, not NIH)

Bacteriophages Isolated from Group B Streptococci

As an extension of prior and ongoing investigations of lysogeny among streptococci, 7 strains of serologic Group B streptococci were treated with mitomycin C and examined by electron microscopy for induced bacteriophages. Only one strain was positive, but two others were good indicators--making possible propagation and further study of the phage. Although morphologically identical (a long-tailed type B phage) to all those temperate phages found previously in streptococci of Groups A, C, G, and H, the phage from the Group B streptococcus failed to lyse or to infect (by plaque assay) any strains of the other groups. In addition, in contrast to the other phages, it does not require calcium for formation of plaques. It may, therefore, be group-specific, and will be employed in genetic and other studies. No bacteriophages from Group B streptococci, which include 5 serotypes and are an important cause of fatal neonatal meningitis, were previously known.

Extension of studies of Group A streptococci shows that 100, out of 102 strains of 47 different serologic types, carry at least one prophage inducible by mitomycin C. Six such phages, picked at random, were all different by serological tests. (Colon, Cole, Popkin)

Relation of Intracellular and Extracellular Hemolysins of Group A Streptococci

Previous work showed that Group A streptococci which make extracellular Streptolysin S (SLS) contain SLS-related cellular hemolysins, including a potential cellular hemolysin (PH). This year's concentration on PH led to the development of a simpler and more efficient assay system, utilizing glass beads and RNA-core to activate the hemolysin. Further investigations on the cellular location of PH shows that, in all Group A strains tested, the greatest total PH activity is in the membrane fraction, but the highest specific activity is usually in the mesosomal fraction. Of the strains tested, nephritogenic streptococci have the highest specific activity in the mesosomes; and, in fact, it appears that nephritogenic and non-nephritogenic streptococci may be distinguishable biochemically by in vitro tests of their PH activities. Additional work done on Streptolysin O (SLO) has shown that this hemolysin is not membrane- nor mesosomal-associated but is periplasmic in location; and that cellular SLO titers do not appear significantly different for rheumatic fever strains. Further efforts are now focused on the pathogenic effects of PH in test animals, with emphasis on the occurrence of renal lesions. (Calandra)

Studies of Gonococcal Infection Initiated

The relation of immune response, and protection or invasion, in gonorrhea is not clear. Studies of clinically acute urethral exudates, to determine the nature and role of immunoglobulins in the urethral environment, are planned. However, the year thus far has required establishment of culture apparatus, media, and conditions; acquisition of reference strains of gonococci and of antisera; culture of the strains; fractionation of antisera; and labelling of antisera for immunoelectron microscopy. Good growth of gonococci was obtained in bi-phasic media, but was inhibited by some lots of Bacto-Agar; only Noble agar is now used. The common problem of variation was encountered, whereby virulent piliated colony types (I, II) give rise to different percentages of conversion to non-virulent types (III, IV). In attempts to avoid this, and still produce rapid and abundant growth in a fluid phase, a new defined medium is currently being tested. A consistent supply of stable colony types is essential to planned experiemnts on interactions of *N. gonorrheae* with human granulocytes and epithelial cells in culture, in the presence and absence of various immunoglobulins and their fractions. (Edmonds, Cole, Popkin)

Cellular Location of Staphylococcal Lipoteichoic Acid Studied

Our previous work on the cellular or subcellular localization of lipoteichoic acid (LTA) indicated its exclusive or predominent association with the mesosome of *Staphylococcus aureus*. The conclusion was based on amounts of LTA extractable from isolated mesosomal vesicles, and little or none was found in media or in other subcellular fractions. Recent work (some still unpublished) from other laboratories indicate that the LTA of some other Gram-positive bacteria is associated with membrane and periplasm (i.e., between membrane and wall), and may penetrate the wall and also be found in large amounts in culture fluid--but not in mesosomes. One proffered explanation, based on results with a *Streptococcus fecalis* system in the virtual absence of magnesium ion (Mg++), is that liberated LTA binds to mesosomal vesicles during isolation in proportion to increasing levels of Mg++. We had used a concentration of 20 mM Mg++, which produced maximal (i.e., essentially 100%) LTA association with mesosomal vesicles. On re-examination of this question, we found that levels as low as 2 mM and as high as 40 mM Mg++ still showed 75% of the 20 mM association level. In the absence of Mg++, the level was still 55%. Variations of osmotic stabilizer (sucrose instead of NaCl) or of lipid extractants (chloroform-methanol instead of pyridine-acetic acid-butanol) did not alter the amounts of LTA extractable by phenol from the mesosomal vesicles. Current studies are re-examining the culture fluids for LTA. The problem is still unsolved, but the supposition must be either that LTA is not localized to the same subcellular sites in different bacteria; or that as yet undefined variations in techniques result in artefactual binding of LTA to, or artefactual liberation of LTA from different sites or subcellular fractions. The natural location is best resolved by *in situ* immunoelectron microscopy of sectioned *S. aureus*; such studies are in progress, but have been delayed by difficulties in reagent preparation. Until subcellular, or other, localization of LTA is clear, reasonable hypotheses on its function in bacteria cannot be established. (Theodore, Huff, Beining, Cole)

Staphylococcal Lipoteichoic Acid As Diagnostic Antigen

Lipoteichoic acids (LTA) are glycerol phosphate polymers occurring in or on mesosomes (intracellular membranous organelles), as well as in other locations in *Staphylococcus aureus* and other Gram-positive bacteria. They can stimulate specific antibodies in experimental animals. S. aureus also possesses a cell-wall teichoic acid (TA), a ribitol phosphate polymer that is also immunogenic in its native (cell wall-associated) state. To determine the occurrence of antibodies to LTA and TA in human infections, sera from 17 patients with staphylococcal endocarditis and 17 uninfected controls were examined. All sera were tested against LTA and TA by precipitin reactions in agar (Ouchterlony). Anti-LTA was also assayed by hemagglutination of LTA-coated erythrocytes. In addition, sera were tested for agglutination of purified cell walls of S. aureus, strain Lafferty--from which strain the LTA and TA were also prepared. Ninety percent of endocarditis sera precipitated LTA, and no control sera were positive. Precipiting tests for TA were less sensitive, as were the other tests--which were also less specific. The precipitin test of LTA thus offers useful diagnostic possibilities, but is under continued investigation because the LTA antigen differs in reactivity according to source. LTA prepared from disrupted whole cells is reactive, whereas that from isolated mesosomes is not. Studies of chemical differences of the two preparations are in progress. (Huff, Theodore, Cole; Sheagren and Tuazon, not NIH)

Factors Relating to Genetic Exchange Among Streptococci Further Defined

Recent studies demonstrated that in Streptococcus sanguis (Wicky) cells, competence factor (CF) induces a minimum of two new proteins. One is manifested as a band after subjecting acid-phenol extracts of cells to acid-gel polyacrylamide-gel-electrophoresis (PAGE). The other is manifested as an autolysin of limited activity; it acts only at the cross-wall region. General inhibitors of protein synthesis, i.e., chloramphenicol, rifampin, prevent the induction of both proteins whereas mercuric chloride or N-ethyl-maleimide prevent the induction of the PAGE protein. This result shows that the two proteins are not one-and-the-same. However, both are essential for the attainment of the competent state. Since recombination of deoxyribonucleic acid (DNA) molecules must occur to complete the transformation process, and since an ATP-dependent exonuclease (Exo-V) has been implicated by others in genetic recombination, this enzyme activity was assayed in both competent and non-competent cell extracts. Equal Exo-V activities were found. It is, therefore, concluded that the PAGE protein is not related to Exo-V activity, and that CF does not induce Exo-V. The function of the PAGE protein remains to be elucidated. In addition to Exo-V, extracts derived from competent and non-competent cells contained another enzyme that degrades denatured (single stranded) DNA in the absence of ATP. Neither of these enzymes have heretofore been described in cells of Streptococcus sanguis. Future research will include examining cultures for DNA restricting enzymes and DNA modifying enzymes, in addition to determining a function for the PAGE protein. (Ranhand)

Administrative, Organizational, and Other Changes

The Laboratory of Streptococcal Diseases completed its second year of existence, but only its first year in the absence of the prior Laboratory of Microbiology. As the latter ceased to exist as an entity on June 30, 1974, its personnel became variously distributed. Dr. Theodore S. Theodore, Dr. Eskin Huff, and Mr. Willard Davis were transferred to LSD. Dr. Arthur Schade retired from the Commissioned Corps, U.S.P.H.S. Dr. Herbert H. Hasenclever was reassigned to the Rocky Mountain Laboratory. Drs. Roy Repaske and

Sanford H. Stone were reassigned to the Office of the Scientific Director, NIAID.

Within LSD, Mrs. Carmen G. Leonard unexpectedly retired on June 1974, thereby decreasing our representation in streptococcal genetics. On July 1, 1974, Dr. Harvey L. Edmonds entered on duty as Research Associate, replacing Dr. Kenneth M. Nugent. On June 30, 1975, Dr. Gary B. Calandra, Research Associate, will leave for extended in-service training in clinical medicine and infectious diseases at Parklawn Hospital, Dallas.

The year was marked by turmoil, uncertainty, and a reduction in productivity--all occasioned by remodeling and a subsequent extended relocation of personnel, equipment, and offices from Building 5 to Building 7, and some moves within Building 7.

The mission of the Laboratory of Streptococcal Diseases is the advancement of knowledge of streptococci, and of other microorganisms, which may contribute to an understanding of genetic mechanisms, pathogenesis, and the relationships of structure and function of bacterial cell walls, membranes, organelles, biologically active molecules or aggregates, and bacteriophages.

Honors and Awards

Roger M. Cole

Member, Editorial Board, Infection and Immunity.

Member, Editorial Board, Antimicrobial Agents and Chemotherapy.

Chairman, Ultrastructure Division, American Society for Microbiology.

Lecturer: "Bacterial Membranes and Mesosomes," in Graduate Seminar Series on Biological Membranes; sponsored by the Consortium of Universities and the Smithsonian Radiation Biology Laboratory, Washington, D. C., March 20, 1975.

Project Officer: NIAID Research Contract PH 43-68-83, University of Chicago.

Invited participant (presentation):

1. 2nd European Meeting on Transformation and Transfection in Microorganisms, Cracow, Poland, Sept. 3-6, 1974. Presentation: "Transduction in Group H Streptococcus." A. E. Colon, R. M. Cole, C. L. Parsons, and C. G. Leonard.

2. International Congress on Les Mycoplasmes de l'Homme, des Animaux, des Vegetaux, et des Insectes, Bordeaux, France, Sept. 11-17, 1974. Sponsored by INRA-INSERM. Presentation: "Virus-like particles in Spiroplasma citri." R. M. Cole, J. G. Tully, and T. J. Popkin.

3. Workshop-Symposium: Fluorescent Antibody and Immunochemical Methodologies Used in the Identification of Cariogenic Bacteria. Sponsored by National

Institute of Dental Research, New York Hilton, April 3-4, 1975. Presentation: "Attributes of potential utility in differentiating among "Group H" streptococci or Streptococcus sanguis." R. M. Cole, G. B. Calandra, E. Huff, and K. M. Nugent.

4. Seminar on The Mesosome--Ultrastructural and Biochemical Organization and Its Significance in Cell Physiology, Annual Meeting of the American Society for Microbiology, New York, April 27 - May 2, 1975. Presentation: Opening Remarks--"What do we mean by'mesosome'?"

5. Seminar on Spiroplasmas: Helical Wall-Free Prokaryotes. Annual Meeting of the American Society of Microbiology, New York, April 27 - May 2, 1975. Presentation: "Virology of Spiroplasmas."

Eskin Huff

Co-author on invited paper on "Chemical Composition and Functional Activities of Mesosomes of Staphylococcus aureus." (T. S. Theodore and E. Huff) at the Annual Meeting of the American Society for Microbiology in New York, April 27 - May 2, 1975.

Jon M. Ranhand

Lecturer, NIH Graduate School, Foundation for Advanced Education in the Sciences.

Participant at the XVIII Annual Meeting on Microbial Transformation, Durham, New Hampshire, June 1974.

Theodore S. Theodore

Invited participant at seminar on the Mesosome--Ultrastructural and Biochemical Organization and Its Significance in Cell Physiology, Annual Meeting of the American Society for Microbiology, New York, April 27 - May 2, 1975. Presentation: "Chemical composition and functional activities of mesosomes of Staphylococcus aureus."

Member: Management Advisory Committee, NIAID Laboratory and Clinical Research

Serial No. Z01 AI 00001-14 LSD
1. Streptococcal Diseases
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemical Studies on Staphylococcal and Streptococcal Membranes, Cell Walls, and Mesosomal Vesicles.

Previous Serial Number: 62

Principal Investigator: Dr. Eskin Huff

Other Investigators: Dr. Theodore S. Theodore
Dr. Roger M. Cole

Cooperating Units: Dr. John M. Sheagren and Dr. Carmelita U. Tuazon
Howard University Medical Service, D. C. General
Hospital, Washington, D. C.

Man Years:	NIAID Lab. Staff	Other NIH Personnal Assigned to Project	Guest Workers	TOTAL
Total	21/12	-	-	21/12
Professional	10/12	-	-	10/12
Other	11/12	-	-	11/12

Project Description:

Teichoic acids (polyribitol phosphates) are immunologically important antigens occurring in the cell walls of S. aureus and other Gram-positive organisms. The purpose of this study has been: 1) to determine the exact mechanisms involved in the biosynthesis of the teichoic acids; 2) to relate the occurrence and nature of teichoic acids to the clinical diagnosis of staphylococcal infection.

We reported last year that lipoteichoic acid (LTA), a glycerol phosphate polymer which reportedly occurred in bacterial membranes, was localized on mesosomes (intracellular membranous organelles) in S. aureus (Huff et al, J. Bacteriol. 120, 1974). Fiedler and Glaser (J. Biol. Chem. 249, 1974) show that LTA in S. aureus acts as a template for the synthesis of a polymer (LTA-TA) in which LTA is attached to TA. This molecule is presumably an intermediate in the synthesis of cell wall teichoic acid. In their enzyme preparation from "membranes" these authors did not distinguish between mesosomes and membranes. We wish to test for the synthesis of LTA-TA by both mesosomes and membranes in order to determine if mesosomes, as indicated by their high levels of LTA, are of primary importance in the synthesis of cell wall teichoic acids. In this regard we are currently involved in the chemical synthesis of cytidine diphosphate (C^{14}) ribitol, the precursor for synthesis of TA.

S. aureus, and all other Gram-positive bacteria studied except M. lysodeikticus, contain lipoteichoic acids. A study has been carried out to determine whether lipoteichoic acid antibodies are produced in humans during infection with S. aureus. In preliminary experiments we found that mesosomes from S. aureus could produce high LTA antibody titers (as demonstrated by complement fixation) when injected into rabbits. We examined a series of 17 patients with staphylococcal endocarditis and 19 non-infected controls for the presence of LTA antibodies. Crowder and White have reported that antibodies to staphylococcal cell wall TA (not found usually in normal sera) are produced in patients with staphylococcus endocarditis. Therefore, we have also tested all sera for cell wall TA antibodies. Antigens have been prepared from S. aureus, Lafferty, a coagulase-positive strain (phage type 52/42B/80/81), lysates of which Crowder and White (Ann. Int. Med. 77, 1972) used to test for cell wall TA by precipitin band formation in agar double diffusion. We have also prepared lysates of cell walls of this organism using lysostaphin. Mesosomes, cell walls, membranes and LTA were prepared as described in previous reports. LTA was prepared from mesosomes and from disrupted cells after removal of cell walls. Sera were tested for LTA antibodies using precipitin reaction by agarose double diffusion and also by hemagglutination of red cells coated with lipoteichoic acid from L. casei. Other tests run were: 1) precipitins for cell wall TA by double diffusion in agarose; 2) agglutination of Lafferty cell walls. When tested against human sera from patients with staphylococcal endocarditis, the lipoteichoic acid preparation from cell contents gave a positive precipitin band with 90%: no sera from non-infected controls gave positive reaction. The TA precipitin test was not as sensitive, and the other tests neither as sensitive nor as specific as the LTA precipitin test. We are presently investigating the chemical structure of the LTA from cell contents since it differs from LTA isolated from the mesosome: the later does not react in the precipitin reaction.

We propose to continue to (1) identify immunologically active materials found in Streptococcus and Staphylococcus species; (2) to determine the biochemical steps involved in the synthesis of TA and LTA in S. aureus.

Keyword Descriptors:

Mesosomal lipoteichoic acid
Staphylococcal infection
Immunological diagnosis
Cell Wall teichoic acid
Precipitin tests
Bacterial antigens
Antibodies
Endocarditis

Publications:

Huff, E., Cole, R. M., and Theodore, T. S.: Lipoteichoic acid localization in mesosomal vesicles of Staphylococcus aureus. J. Bacteriol. 120: 273-281, 1974.

Nugent, K. M., Huff, E., Cole, R. M., and Theodore, T. S.: Cellular location of degradative enzymes in Staphylococcus aureus. J. Bacteriol. 120: 1012-1016, 1974.

Beining, P. R., Huff, E., Prescott, B., and Theodore, T. S.: Characterization of the lipids of mesosomal vesicles and plasma membranes from Staphylococcus aureus. J. Bacteriol. 121: 137-143, 1975.

Theodore, T. S., Cole, R. M., and Huff, E.: Localization of glycerol phosphate in mesosomal vesicles of Staphylococcus aureus. Biochem. Biophys. Res. Comm. 59: 215-220, 1974

Serial No. Z01 AI 00002-10 LSD
1. Streptococcal Diseases
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the Nutrition, Physiology, and Ultrastructure of *Staphylococcus aureus*

Previous Serial Number: 63B

Principal Investigator: Dr. Theodore S. Theodore

Other Investigators: Dr. Eskin Huff
Dr. Roger M. Cole
Dr. Paul Beining, Dept. of Biology, University of Scranton, Scranton, Pa.

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	27/12	-	3/12	30/12
Professional	15/12	-	3/12	18/12
Other	12/12	-	-	12/12

Project Description:

The membrane system of *S. aureus* and other Gram positive bacteria consists of the plasma membrane and mesosomes (intracytoplasmic membranous organelles). The objective of this study is to isolate the two membrane fractions and analyze their ultrastructure, chemical composition and enzymatic activities in an attempt to determine the function (or functions) of mesosomes. Last year we reported on the nearly exclusive localization of lipoteichoic acid (LTA) of *S. aureus* in mesosomes and showed the predominant site of this polyglycerol phosphate polymer to be mesosomes of other Gram positive bacteria. However, these findings conflict with other reports in which LTA of other Gram positive bacteria has been associated with the plasma membrane, periplasmic fraction and the cell surface. To establish more precisely the location of LTA in mesosomes of *S. aureus*, various aspects of protoplast formation, mesosome isolation and LTA extraction were examined. Protoplasts were prepared in the presence of varying Mg^{++} concentrations which are known to affect LTA binding. Ranges between 2 and 40 mM had little effect on protoplast formation, release of mesosomes or on the localization of LTA. At 2 and 40 mM Mg++ the LTA bound to mesosomes was approximately 75% of that found under normal conditions (20 mM). The amount of LTA present in membranes at any Mg++ concentration was minimal. When protoplasts were prepared in the absence of Mg++ (conditions known to affect protoplast formation and release of mesosomes), LTA was still associated with the mesosomes. The amount present was approximately 55% of that normally found. The substitution of 20% sucrose for NaCl as an osmotic stabilizer during protoplast formation; and the extraction of lipids with chloroform/methanol (2/1) rather than pyridine/acetic acid/butanol (22/31/100) did not alter the mount of LTA that could be extracted by aqueous phenol (40%). The projected use of LTA antisera for the *in situ* localization of LTA by ferritin-labelled antibody techniques was unsuccessful due to the low titer of the antisera prepared.

Amino acid characterization of the mesosomal extracted LTA showed that approximately 86% of the amino acid content of lipid-depleted, aqueous phenol extracted mesosomes was alanine. Polyacrylamide gel electrophoresis on the aqueous phenol extracted LTA showed a very homogeneous pattern with only one protein band which stained positively with periodic acid-Schiff reagent. Aqueous phenol extracted membranes were devoid of any bands.

Serological data pertaining to the *in situ* location of LTA is still lacking, but the localization by chemical means appears to be well established. However, there is still no definitive evidence as to the functional role of mesosomes. Recently it has been reported that LTA is synthesized by the turnover of phosphatidyl glycerol and that the LTA functions as an acceptor molecule in the synthesis of wall teichoic acid (Glaser and Lindsay, 1974, BBRC, 59, 1131; Fiedler and Glaser, 1974 JBC, 249, 2684). These findings are consistent with our already published results (see publications #6 and #4 below) on the 6 fold increased level of phosphatidyl glycerol and LTA localization in mesosomal vesicles and strongly suggest mesosomal involvement in LTA and cell wall synthesis.

The planned course of this project will include: 1) new immunizations of rabbits with new and immunogenic preparations of LTA; 2) immunoelectron microscopic localization of LTA in intact cells of *S. aureus*; 3) characterization of the chemical structure of *S. aureus* LTA; and 4) analysis of the steps involved in the biosynthesis of LTA and its possible function.

Keyword Descriptors:

Staphylococci
Mesosomes
Membranes
Lipoteichoic acid
Magnesium ions

Publications:

1. Tully, J. G., Barile, M. F., Edwards, D. G. ff, Theodore, T. S., and Erno, H.: Characterization of some caprine mycoplasmas, with proposals

for new species *Mycoplasma capricolum* and *Mycoplasma putrefaciens*. *J. Gen. Microbiol*. 85: 102-120, 1975.

2. Theodore, T. S., and Weinbach, E. C.: Respiratory activities associated with mesosomal vesicles and protoplast membranes of *Staphylococcus aureus*. *J. Bacteriol*. 120: 562-564, 1974.

3. Theodore, T. S., Cole, R. M., and Huff, E.: Localization of glycerol phosphate in mesosomal vesicles of *Staphylococcus aureus*. *Biochem. Biophys. Res. Comm*. 59: 215-220, 1974.

4. Huff, E., Cole, R. M., and Theodore, T. S.: Lipoteichoic acid localization in mesosomal vesicles of *Staphylococcus aureus*. *J. Bacteriol*. 120: 273-281, 1974.

5. Nugent, K. M., Huff, E., Cole, R. M., and Theodore, T. S.: Cellular location of degradative enzymes in *Staphylococcus aureus*. *J. Bacteriol* 120: 1012-1016, 1974.

6. Beining, P. R., Huff, E., Prescott, B., and Theodore, T. S.: Characterization of lipids of mesosomal vesicles and plasma membranes of *Staphylococcus aureus*. *J. Bacteriol*. 121:137-143, 1975.

Serial No. Z01 AI 00003-2 LSD
1. Streptococcal Diseases
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Characteristics of "Group H" Streptococci

Previous Serial Number: 65A-4

Principal Investigator: Dr. Gary B. Calandra
Dr. Roger M. Cole

Other Investigators: Dr. Eskin Huff

Cooperating Units: Streptococcal Laboratory, CDC, Atlanta (Dr. Richard D. Facklam)

Center for Oral Health Research, University of Penn., Philadelphia (Dr. Burton Rosan)

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	6/12	-	-	6/12
Professional	4/12	-	-	4/12
Other	2/12	-	-	2/12

Project Description:

The objectives of this study were to utilize various attributes or characteristics to clarify the relationships of streptococci variously designated as being of serologic "Group H", or Streptococcus sanguis. These bacteria are the most numerous organisms on tooth surfaces and in dental plaque, may cause or aggravate caries, and are a common cause of subacute bacterial endocarditis in man. In addition, the group includes those streptococci subject to DNA-mediated genetic transformation, and we recently reported the existence of "Group H" bacteriophages and phage-mediated genetic transduction.

A collection of 28 "classical" or reference strains was examined for several attributes. By standard physiological tests, all were classifiable as S. sanguis. Antigenic compositions determined by immunoelectrophoresis of autoclaved extracts versus a standard S. sanguis (M5) antiserum separated the strains into Rosan's 3 types, and No Type. The most common was type II (16 strains), followed by type I (9 strains), Heterogeneous (2 strains) and No Type (1 strain). The distribution by type was reflected by patterns of other attributes. These included sensitivity to a muralysin (PAL or phage-associated lysin of Group C streptococci), reactivity of extracts with a "Group H" antiserum (strain Blackburn) and a "non-H" antiserum (strain K208), adsorption of a Group H streptococcal phage, the presence of cell surface fimbriae, and the presence and relative amounts of cell-wall sugars. Type II, Heterogeneous type, and No Type were readily distinguished from one another by distinct and consistent patterns of PAL sensitivity and serological reactivity. However, type I strains were variable in these attributes, and could be separated from those belonging to other types only by patterns of the additional attributes. Phage adsorption was found only in type II; fimbriae in only type I or Heterogeneous type; low cell wall galactose only in type II; low cell wall rhamnose, plus the presence of ribitol, only in Heterogeneous or No Type. It is suggested that the Heterogeneous and No Type strains, which include those previously known as S. sanguis II (based on old serologic data) should no longer be considered as S. sanguis. The data indicate detectable heterogeneity in antigenic and apparently associated other attributes among S. sanguis or "Group H" streptococci that are often used as reference strains, and therefore suggest the utility of such attributes for purposes of screening and taxonomic differentiation.

It is planned to continue this investigation, as time permits, with other strains and other attributes, even though one of the principal investigators (Calandra) will leave at the end of the fiscal year.

Keyword Descriptors:

Streptococcus sanguis
Muralysin (cell wall-splitting enzyme)
Serological classification
Dental caries
Bacterial endocarditis
Cell wall sugars
Physiological tests
Group H streptococcal bacteriophage

Publications:

Calandra, G. B., Nugent, K. M., and Cole, R. M.: Preparation of protoplasts of Group H streptococci (Streptococcus sanguis). Appl. Microbiol. 29: 90-93, 1975.

Cole, R. M., Calandra, G. B., Huff, E., and Nugent, K. M.: Attributes of potential utility in differentiating among "Group H" streptococci or Streptococcus sanguis. J. Dental Res. (Supplement): (in press)

Serial No. Z01 AI 00004-15 LSD
1. Streptococcal Diseases
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Electron Microscopy of Bacteria in Relating Structure and Function

Previous Serial Number: 65

Principal Investigator: Dr. Roger M. Cole

Other Investigators: Mr. Terry J. Popkin
Dr. Jon M. Ranhand

Cooperating Units: University of Arizona, College of Liberal Arts, Dept. of Microbiology and Medical Technology, Tucson (Dr. Neil H. Mendelson)

Dr. J. G. Tully (Serial No. Z01 AI 0027-8 LID)

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	21/12	-	-	21/12
Professional	10/12	-	-	10/12
Other	11/12	-	-	11/12

Project Description:

Electron microscopy (EM) is a tool that supplies information not obtainable by other means, and that supplements other types of information. The objective of this project is to apply this tool to primary studies of the ultrastructure of microorganisms, and to other investigations in which ultrastructural information is an important aspect--supplementary to data on function or chemical composition, for example, but not the prime feature. The balance between these types of studies varies from year to year, and the reporting of results does not lend itself to quite the same format nor unification as many other projects.

In the past year, for example, much time was spent on examination of different strains of a mycoplasma (Spiroplasma citri): this resulted in discovery and morphologic description of 3 new viruses, and might be considered as a "primary study" up to this point. However, since the study has now become more concerned with the occurrence, geographic distribution, cultural stimuli for production, and propagation of these viruses, it seemed more appropriate to transfer it to a separate new project with a broader scope (See Project No. Z01 AI 00007-1 LSD).

"Supportive" type studies are usually and eventually reported in detail under the appropriate related project, and it continues to be useful, in order to present the contributions of this project, to list them briefly and individually with cross-referencing where appropriate.

1. Streptococci of Groups A and B were monitored for the EM detection of bacteriophage after induction procedures. (Project No. Z01 AI 00005-10 LSD, Colon)

2. EM cytochemical examination of Group A streptococci in section was made to localize acid and alkaline phosphatases. Initial results were equivocal, and are being repeated. (Project No. Z01 AI 00009-2 LSD, Calandra)

3. Numerous preparations of subcellular fractions of staphylococci and other Gram-positive bacteria were monitored for the presence and purity of mesosomal vesicles, membranes, and other constituents. (Project Nos. Z01 AI 00001-14 LSD, Huff; Z01 AI 00002-10 LSD, Theodore; Z01 AI 00003-2 LSD, Calandra)

4. Gonococci were examined by negative stain for verification of colonial (virulence) type by presence of pili; and by section for general ultrastructural features--preliminary to experiments on immunoglobulin reactivity and phagocytosis. (Project No. Z01 AI 00008-1 LSD, Edmonds)

5. Gradient-purified Group H streptococcal phage 227, and its DNA in Kleinschmidt spreads, were examined and photographed--the latter for aid in determining DNA molecular weights by contour lengths. (Collaborator, Dr. Garon, LBV) (Project No. NIAID 65A-2, Nugent; this project terminated last June, but the above mentioned work was completed after last year's reporting deadline.)

6. EM, by both negative stain and sections, showed that a toxic strain of Mycoplasma neurolyticum consisted of relatively uniform, spherical, and often empty cells; whereas a non-toxic strain comprised variable, often filamentous, dense cells. Structures suggesting toxin itself, or its cellular location, were not detected. (Project No. Z01 AI 0027-8 LID, Tully)

7. Agrobacterium tumefaciens (a plant bacterium) was monitored for bacteriophage after mitomycin C induction; and purified phage obtained from an outside source was also examined. The purpose was comparison with Spiroplasma citri phages, to see if one plant pathogen produces phages which might contaminate another, or which might be confused with one another. All phages are distinct in size and ultrastructure.

8. EDTA-treated preparations of Gram-negative bacteria--made by Dr. Ranhand--were repeatedly examined to detect, and determine the nature of, small

pseudohelical filaments produced by such treatment. Such filaments are 140 A° in diameter, of different lengths (up to 0.5 um), and appear composed of small subunits arranged in a 6 or 7-membered hollow stellate disc. They appear to originate from cytoplasmic membrane, rather than from outer membrane as do simultaneously-released lipopolysaccharide vesicles. Because they are unstable during separation procedures, or on storage under the variety of conditions tested so far, quantities for chemical assay have not been accumulated and EM monitoring is the only means of detection. Their nature and significance is therefore unknown. They have been found in preparations of several strains of E. coli, Salmonella, Shigella, Serratia, Klebsiella, Proteus, and Proteus L forms; but not in Gram-positive bacteria, nor in Pseudomonas, Aerobacter, Alkaligenes, Neisseria gonorrheae, nor Veillionella. However, cultural conditions may affect their appearance, and living intact cells are required for their appearance. As time permits, re-examinations and attempts at stabilization will be made.

9. In a collaborative attempt to visualize and count the number of genomes (DNA, or nucleoids) per cell of a mutant of Bacillus subtilis, the bacteria were treated successively with hot sodium dodecyl sulfate (SDS), pronase, RNAase, and DNAase, and examined by both phase microscopy (PM), and EM. SDS produced a "barred" pattern by PM, which EM showed to consist of alternating blocks of coagulated cytoplasm and of granular areas. The former disappear after pronase, but are replaced large membranous vesicles containing crystalline bodies and numerous small membranous bodies: the interpretation is that pronase removed much protein, but that membrane components disaggregated by SDS were reaggregated after washing out of SDS and exppsure to magnesium ions in the pronase-buffer mixture. By PM, dark granules and lighter areas are seen. By successive RNAase and DNAase treatments, the original granular areas were shown to contain RNA and DNA, but the distinguishability of these by PM is not as useful a marker as anticipated. (Collaborator, Mendelson, University of Arizona)

We expect to continue ultrastructural studies as indicated in attempting to provide subcullular bases for, or associations with, basic microbiological phenomena related to genetics, bacterial growth and division, the structural localization of chemical components and activities, and pathogenesis and virulence.

Keyword Descriptors:

Electron microscopy
Bacterial structure and function
Streptococci
Bacilli
Gram-negative bacteria
Bacteriophage
Subcellular fractions
Cytochemistry

Publications:

Huff, E., Cole, R. M., and Theodore, T. S.: Lipoteichoic acid localization in mesosomal vesicles of Staphylococcus aureus. J. Bacteriol. 120: 273-281, 1974.

Theodore, T. S., Cole, R. M., and Huff, E.: Localization of glycerol phosphate in mesosomal vesicles of Staphylococcus aureus. Biochem. Biophys. Res. Comm. 59: 215-220, 1974.

Nugent, K., Huff, E., Cole, R. M., and Theodore, T. S.: Cellular location of degradative enzymes in Staphylococcus aureus. J. Bacteriol. 120: 1012-1016, 1974.

Calandra, G. B., Nugent, K. M., and Cole, R. M.: Preparation of protoplasts of Group H streptococci (Streptococcus sanguis). Appl. Microbiol. 29: 90-93, 1975.

Cole, R. M., Tully, J. G., and Popkin, T. J.: Virus-like particles in Spiroplasma citri. Des Colloques de L'I.N.S.E.R.M. 33: 125-132, 1974.

Cole, R. M., Calandra, G. B., Huff, E., and Nugent, K. M.: Attributes of potential utility in differentiating among "Group H" streptococci or Streptococcus sanguis. J. Dental Res. (In press)

Serial No. Z01 AI 00005-10 LSD
1. Streptococcal Diseases
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on Bacteriophages and Genetics of Streptococci

Previous Serial Number: 65A

Principal Investigator: Alba E. Colón

Other Investigators: Dr. Roger M. Cole
Mr. Terry J. Popkin

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	21/12	-	-	21/12
Professional	12/12	-	-	12/12
Other	9/12	-	-	9/12

Project Description:

The objective of this project is to study the role of lysogeny and genetic exchange in streptococci (transduction, transformation, and lysogenic conversion) and to try to elucidate how such mechanisms may affect important attributes such as antigenic characteristics, virulence or pathogenic factors.

Last year we studied the extension of lysogeny in Group A streptococci. This knowledge was a requisite as it is well known that lysogeny can alter host susceptibility to phage adsorption, produce immunity to isolated phages and affect directly the characteristics of the cells. At the time we reported, 100% lysogeny among 40 randomly chosen strains representing various M protein types, "classics" as well as old and new isolates. This high percentage of lysogeny was obtained by direct electron microscopic (EM) examination of mitomycin C induced preparations.

This year we extended our investigation to a total of 102 strains, representing 47 different serological M types, plus untypable strains. All were found to be lysogenic except for 2 out of 4 M-type 23 strains. Repeated EM examination of UV irradiated as well as mitomycin C induced preparations of these two strains failed to show any bacteriophages. In addition, the induced supernatants also failed to plaque or lyse any of 5 indicator strains tested. All phages were morphologically of Bradley's Group B and they appear to be complete infecting particles. Induced supernatants of 30 strains chosen at random were spotted on possible indicator lawns. All of them lysed, or plaqued on, one or more of the tested strains.

Six strains were picked for further investigation. The phages were isolated and propagated to high titers. Serological identification showed they were antigenically different from each other as well as from a previously isolated virulent Group A phage.

Work is now in progress in attempting to "cure" these six strains of their prophage. The "cured" strains will be tested for antigenic changes, ability to be transducing donors and ability to transform. Conversely, any changes in attributes after acquisition of a prophage by the cured strains (lysogenic conversion) will also be investigated.

In addition, using a bacteriophage isolated from a Group G M-type 12 strain, attempts will be made to transduce the M-protein or cell wall carbohydrate markers from that strain to another Group G or Group A strain.

Group B streptococci have become increasingly significant because of their pathogenicity in infants. There is no report in the literatures on the occurrence of bacteriophages in these streptococci. Using the same techniques as described for Group A, 7 Group B strains were studied. Bacteriophage was found in one of the strains, while two other strains were good indicators. The phage morphologically belonged to Bradley's Group B, and unlike Group A phage, it does not require $CaCl_2$ for plaque formation. It also appears to be group specific because it did not lyse or plaque in any of the Group A, C, G, and H strains used as indicators.

The significance of these studies is that the field of streptococcal genetics is relatively new and little is known concerning the genetic exchange in naturally occurring populations of Group A streptococci. Additional knowledge may provide a better understanding of the variability and pathogenicity of these organisms.

Keyword Descriptors:

Group A streptococci
Group B streptococci
Transduction
Transformation
Lysogenic conversion

Publications:

None

Serial No. Z01 AI 00006-4 LSD
1. Streptococcal Diseases
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Competence Development and Genetic Exchange Mechanisms in Streptococci.

Previous Serial Number: 65C-1

Principal Investigator: Dr. Jon M. Ranhand

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	12/12	-	-	12/12
Professional	12/12	-	-	12/12
Other	-	-	-	-

Project Description:

My long term objectives are to describe the phenomenon of genetic transformation and its ramifications in Streptococcus sanguis cells. These include competence development, deoxyribonucleic acid (DNA) uptake, recombination, and expression of newly acquired genes.

In Escherichia coli cells, an ATP-dependent DNAse (Exo-V; Rec-B, Rec-C) has recently been shown to play an essential role in gene recombination. Since transformation involves gene recombination, and since competence factor (CF) induces a number of new proteins (see earlier reports) it was of interest to determine whether CF induced Exo-V-like activity in Streptococcus sanguis cells. Extracts prepared from CF induced and CF non-induced cultures revealed that Exo-V-like activity was present equally in both. The above extracts also contained another DNAse similar in activity to Exo-I; an enzyme that degrades denatured (single stranded) DNA to acid soluble products in the absence of ATP. This enzyme too, is thought to play a role in recombination. Neither of these enzymes have heretofore been described in Streptococcus sanguis. Work should now progress in defining these nucleases as well as investigating whether streptococci contain DNA restricting and DNA modifying enzymes.

Examination of cultures for enzymes has been greatly facilitated by my recent discovery that now permits these relatively hard-to-break cells to be disrupted by a rather simple procedure. This procedure involves vortexing cells in the presence of small glass beads. Depending on the cells used, breakage ranged from 48 to 92% after 2 minutes of treatment. The convenience and simplicity of the method allows for easy survey of the progress of breakage, the maintenance of reduced temperatures, and the maintenance of the gas phase above the samples. Other assets for the procedure are (i) reduced aerosols, since breakage is done in screw-capped tubes, (ii) the glass beads can be sterilized by autoclaving them *in situ*, and (iii) for large number of cultures, cells can be grown and broken in the same tube. The method has been used to break Gram positive cells (streptococci, staphlococci, lactobacilli, bacilli) and Gram negative cells (Escherichia, Salmonella, Proteus) as well as yeasts and mammalian tissue culture cells.

Continuation of this study will involve examinations for intracellular enzymes which restrict or modify DNA's entering the streptococcus. Such enzymes are known in other bacteria, but have not been investigated in *S. sanguis*; and are of basic importance to genetics of these microorganisms.

Keyword Descriptors:

Streptococcus sanguis
Transformation
Competence
Recombination
DNAse
DNA

Publications:

Ranhand, J. M.: Inhibition of the development of competence in *Streptococcus sanguis* (Wicky) by reagents that interact with sulfhydryl groups. Discernment of the competence process. *J. Bacteriol*. 118: 1041-1050, 1974.

Ranhand, J. M.: Inhibition of transformation by sodium metaperiodate in *Streptococcus sanguis* (Wicky). Demonstration that deoxyribonucleic acid binding sites contain amino acids. *J. Bacteriol*. 118: 1192-1193, 1974.

Ranhand, J. M.: Simple, inexpensive procedure for the disruption of bacteria. *Appl. Microbiol*. 28: 66-69, 1974.

Serial No. Z01 AI 00007-1-LSD
1. Streptococcal Diseases
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Viruses of Spiroplasma citri and other mycoplasmas

Previous Serial Number: None

Principal Investigator: Dr. Roger M. Cole

Other Investigators: William O. Mitchell
Terry J. Popkin

Cooperating Units: Dr. J. G. Tully, (Serial No. Z01 AI 0027-8 LID)

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	22/12	-	-	22/12
Professional	5/12	-	-	5/12
Other	17/12	-	-	17/12

Project Description:

Our objectives are to define and describe several viruses found in Spiroplasma citri, a recently discovered and cultured mycoplasmal plant pathogen. Viruses have been known for several years in Acholeplasma laidlawii, a sterol-non-requiring mollicute, but were unknown in mycoplasmatales (sterol-requiring "true" mycoplasmas) until our discovery, 2 years ago, of a tailed bacteriophage in S. citri. Within the reporting year, we have discovered 2 additional viruses in S. citri strains originating from 5 different laboratories in 4 different countries. All 27 strains examined produce one or more viruses, and many produce, simultaneously, 2 or 3 viruses. Over 400 passages of the several strains, were examined: in one 4-month period, 74% of passages were positive for at least one virus. Many strains are virus-positive as received; most of the remainder are positive on first passage here. This, plus the diverse geographic origins, universal virus presence, sporadic detectability of virus (not seen in every passage), and the finding of intracellular virus stages in sectioned S. citri cells, appears to obviate laboratory contaminations and extraneous bacterial viruses.

The viruses are presently detectable only by electron microscopy and are of 3 morphologic types: SV-C1 (a rod-shaped particle); SV-C2 (a long-tailed type B bacteriophage); and SV-C3 (a smaller, short-tailed bacteriophage). Recent efforts were directed to selection of media for better virus detection; several variations of 4 different media were tested, and one selected which produces 89% virus-positives, of passages examined. These viruses are of importance to basic microbiology for several reasons: (1) no previous instance of tailed bacteriophage-like viruses attacking wall-less bacteria is known; (2) they will offer probes useful to determination of membrane structure, to definition of virus-receptor sites and natures, and to studies of the cycle of virus replication or state (cryptic, or lysogenic) in easily grown cultures of a membrane-bounded microorganism; (3) they may provide data of taxonomic importance by cross-infectivity tests, because other spiroplasmas are now being detected in, and cultured from, other plants and some insects.

Since the viruses are produced sporadically in widely varying numbers, and appear to exist in a state of lysogeny, the greatest current problem is determination of a means of induction of large numbers and the discovery of a suitable indicator strain for virus propagation. Present and future efforts are directed to this end by use of known inducing agents, cross-testing of all strains on all other strains of _S. citri_ for formation of virus plaques, and extension of infectivity testing to other cultivable species of spiroplasma as they become available.

Keyword Descriptors:

Mycoplasma
Spiroplasma citri
Virus
Bacteriophage
Plant pathogen
Membrane structure
Lysogeny

Publications:

Cole, R. M., Tully, J. G., and Popkin, T. J.: Virus-like particles in _Spiroplasma citri_. _Des Colloques de L' I.N.S.E.R.M._ 33: 125-132, 1974.

Serial No. Z01 AI 00008-1 LSD
1. Streptococcal Diseases
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on Pathogenesis in Gonococcal Infection

Previous Serial Number: None

Principal Investigator: Dr. Harvey L. Edmonds

Other Investigators: Dr. Roger M. Cole
Mr. Terry J. Popkin

Cooperating Units: None

Man Years	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	14/12	-	-	14/12
Professional	13/12	-	-	12/12
Other	1/12	-	-	1/12

Project Description:

The purpose of this project is clarification of the host parasite relationship in gonococcal urethritis. By studying clinically acute urethral exudate we will attempt to: determine the distribution of immunoglobulins within host cells as well as the surrounding milieu; contrast the presence of secretory with 7s immunoglobulins; compare the extent of antibody binding to piliated, non-piliated, and cell wall deficient organisms; and finally to learn the extent to which immunoglobulin binding coincides with invasion of host cells and/or phagocytosis.

Clincial isolates are to be screened by gram stain, but identified by oxidase reaction and acid production from glucose, but not lactose, galactose, or maltose. Localization of IgG, IgM, 7s IgA, 11s IgA globulins and C_3 in clinical specimens will be by indirect immune electron microscopy. Ferritin conjugated antibody is being prepared in the laboratory. IgG fractions of antisera were prepared and further purified by immunoadsorption. Ferritin is being conjugated to purified IgG with glutaraldehyde. The conjugate is to be isolated by gel filtration on Agarose 4B. In some studies host cells will be broken by physical methods to provide access of the tracer to intracellular sites.

But, before beginning the immunochemical preparative work now under way or the clinical study that is to follow, it was necessary to set up a culture system for the propagation of Neisseria organisms. In addition, a complex twin source illumination system for light microscopic colony typing was devised and built. Organisms were also viewed electron microscopically in negatively stained and thin sectioned preparations. With N. gonorrhoeae strain F-62 supplied by the CDC, liquid phase culture was performed using GC Agar Base and Dextrose Starch Biphasic medium. The purity of agar used was found to be quite important, as certain lots of Bacto Agar inhibited growth completely. Subsequent media have been made with Noble Agar. An 18 hour culture of type II organisms contained 10^9 CFU/ml with only 10% conversion to other colony types using the biphasic medium. As others have found, this could not be duplicated with colony types I, III, and IV. In attempts to improve and standardize growth, a defined medium originally described by Catlin is now being tried. Sterilization of this medium, though problematic has now been accomplished using pressure filtration through asbestos filter pads. Hopefully, this medium will provide rapid fluid phase growth with stable morphology which may be of great importance in phagocytosis experiments that utilize similarly defined tissue culture media.

Elevations of serum IgG with variable anti-gonococcal specificity and raised content of IgA in urethral secretion have been reported in acute gonorrhea. Though cell wall deficient organisms have been described in urethral pus, the modes of their formation remain unidentified. The comparative importance and efficiency of IgG versus 11s IgA in this disease is unclear. A proteolytic enzyme capable of cleaving F_c fragments from both 7s and 11s species of the IgA_1 allotype have recently been described in culture filtrates from N. gonorrhoeae and N. meningitidis strains. Its presence and role in vivo remain to be explored, though a similar system in Streptococcus sanguis is thought to shield it in part from antibody mediated immunity. The fastidious growth pattern of the pathogen, and its narrow host range point strongly to the presence of successful adaptation by the parasite. The topographic localization of immunoglobulins in infected fluid would seem to be an important guidepost in our understanding of human immune ineffectiveness as it relates to the dynamics of the host parasite relationship.

In addition to the completion of the present studies, a comparison of the interaction of N. gonorrhoeae with human granulocytes and epithelial cells in culture is to be made. Special emphasis is to be made on the kinetics of killing and on intracellular viability over time.

Keyword Descriptors:

Gonorrhea
Pathogenesis
Immune electron microscopy
IgG
IgA
Opsonins
Bacteriolysis
Secretory immunity

Publications: None

Serial No. Z01 AI 00009-2 LSD
1. Streptococcal Diseases
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Investigation of the Streptolysin S-related Cellular Hemolysins of Group A Streptococci

Previous Serial Number: 65A-3

Principal Investigator: Dr. Gary Calandra

Other Investigators: Dr. Theodore S. Theodore

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	23/12	-	-	23/12
Professional	10/12	-	-	10/12
Other	13/12	-	-	13/12

Project Description:

This work was undertaken to determine the temporal and structural relationships among the Streptolysin S(SLS)-related cellular hemolysins and the possible importance of these hemolysins in streptococcal pathogenesis.

The development of a simplified method of activating cellular SLS (known as potential hemolysin or PH) has made the assay of PH easier and less time consuming. PH is activated by vortexing suspect cellular fractions with glass beads and RNA-core: the method is much more efficient than the previously used sonication-activation method, and results in much higher titers.

By this method, the cellular location of PH has been investigated in numerous additional strains of known pathogenic associations. Although the relative amounts of PH in the periplasm, mesosomal fraction, cytoplasm, and membrane vary from strain to strain, the greatest total activity is always in the membrane, while the mesosomal fraction usually has the highest specific activity. Several strains have very high titers of PH in the membranes and may be useful in attempted determination of the composition of PH and SLS.

The cellular location of streptolysin O (SLO)--another cellular hemolysin, but unrelated to SLS--was also examined. Unlike SLS this hemolysin is not associated with the membrane or mesosomal fractions. Alhtough SLO is a cardiotoxin, no significant differences in cellular titers of SLO were found between rheumatic fever strains and non-rheumatic fever strains. In the course of this work, the locations of other cellular enzymes were also assayed as controls. Interestingly and unexpectedly most of the alkaline phosphatase activity was located in the cytoplasmic fraction, rather than in the periplasm as in other bacteria: this finding is unexplained at present. Aldolase and maltase were cytoplasmic, while acid phosphatase was periplasmic, membrane, and mesosomal in location, as expected.

The most interesting and perhaps most important finding is that nephritogenic and non-nephritogenic streptococci can be grouped on the basis of their mesosomal SLS specific activity and membrane SLS total activity. Nephritogenic streptococci as a group have high PH titers in their membranes and very high specific activity of PH in the mesosomal fraction. The respective amounts in non-nephritogenic streptococci are much lower.

The pathogenic effects of PH were examined by injecting membrane and mesosomal fractions containing PH subcutaneously into rabbits. Although such injections resulted in a local delayed inflammatory response, little change in excretion of urinary protein, and no hematuria, occurred--suggesting lack of renal damage. These results are, however, based on small samples.

Although sporadic and infrequent reports of detection of a nephritogenic substance from some Group A streptococci have appeared in the literature, the differences in PH titers of subcellular fractions reported here represent the first instance of a biochemical difference, detectable in vitro, between nephritogenic and non-nephritogenic strains. The higher PH titers in nephritogenic strains suggests that this substance may be involved in post-streptococcal glomerulonephritis: however, there is required a more extensive examination of strains as well as more testing of PH in more appropriate animals. Preliminary tests in rhesus monkeys are in progress, and should aid in determining if PH is directly involved in the pathogenesis of glomerulonephritis, or whether it is simply a marker for an associated substance or activity of primary importance. Additional studies of PH purification and of its effects on known biological systems are needed, but because of lack of other qualified personnel and the June 1975 departure of the Principal Investigator for further clinical training, it will be necessary to terminate the project.

Keyword Descriptors:

Group A streptococcus, Streptococcus pyogenes
Hemolysins - streptolysin S
streptolysin O
Cellular enzymes - phosphatases
Glomerulonephritis
Rheumatic fever

Publications:

None

Serial No. Z01 AI 00060-7 LSD
1. Streptococcal Diseases
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Competence Development and Genetic Transformation in Group H and Group A Streptococci

Previous Serial Number: Same

Principal Investigator: Mrs. Carmen G. Leonard

Other Investigators: Miss Alba E. Colón
Dr. Roger M. Cole
Dr. Theodore S. Theodore

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	-	-	-	-
Professional	-	-	-	-
Other	-	-	-	-

Project Description:

Project has been terminated.

LI

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LBV

LMI

LPD

LABORATORY OF BIOLOGY OF VIRUSES

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Annual Report of the Laboratory of Biology of Viruses
National Institute of Allergy and Infectious Diseases, NIH
July 1, 1974 through June 30, 1975

The Laboratory of Biology of viruses has under investigation biologic phenomena such as transformation of cells by viruses, regulation of viral m-RNA synthesis, mechanisms of DNA replication and synthesis of viral macromolecules. For each of these problem areas the virus and cell system that is used is that which gives the investigation the best chance of sorting things out. Particular viruses, because of size or their site of replication may be particularly suited to the analysis of a specific problem. It is clear, as major problems are better understood, that biologic phenomena in apparently diverse systems have similar explanations at the molecular level. This is true in the area of viral oncology when adenoviruses and SV40 are compared. With greater understanding, viral latency and viral transformation merge and can be seen to have many common features. Studies which have characterized vaccinia viral messenger (m)RNA are seen to apply equally to SV40 and adenovirus mRNA and also to cellular mRNA. In spite of the growing unity of the major areas of research, this annual report divides the work of the LBV into four separate areas. These concern molecular studies with (1) Papovaviruses, (2) Poxviruses, (3) Adenoviruses, and (4) Parvoviruses. The division of the report into these four areas represents a scientifically false separation, but is used as a format for this report since it simplifies the presentation of results. A careful reading of the report will reveal the strength that accrues to the laboratory by an exchange of techniques and a sharing of observations in each of these areas.

The results described below are evidence of solid, well grounded advances in each area of research, and report many new findings of major importance in particular areas.

Studies with Papovaviruses.

There is widespread interest in SV40 since it transforms cells, it is capable of producing tumors _in vivo_ when injected into suitable hosts, and is a virus which has one of the simplest compositions. Its viral genome is a double stranded, covalently closed molecule of 3×10^6 daltons, and so it can direct the synthesis of no more than 5 or 6 viral specified proteins. Largely as a result of work carried out in this laboratory of M. Martin, G. Khoury, N. Salzman and their collaborators, there is a high level of understanding in the way in which synthesis of viral RNA and DNA occurs during a lytic cycle of replication.

This past year, in studies with SV40 mutant viruses, useful new laboratory procedures have been developed. Using agarose gel electrophoresis it was possible to separate virions which contained a temperature sensitive (ts) defect from wild type virions. The ts mutant represents a point mutation in which there is only a single amino acid change in one of the viral proteins; that is, one out of the 750 amino acids in the virion has been changed. While the change in a single protein is small, within the coat of the virus, there are multiple copies of a single protein (as many as 300) and so the effect of a single amino acid change in a protein can be magnified, and produce a change in the charge on the virion surface. Migration of this ts mutant virus as a function of pH initially suggested that the ts change was the consequence of the replacement of a neutral amino acid by a basic amino acid. However, we presently believe that the coat protein may contain a replacement of a neutral amino acid by another neutral amino acid and that this produces a protein with an altered conformation which in turn causes a redistribution of surface charges in the virion. The same technique of agarose electrophoresis which effects a separation of a wild type and a ts mutant of SV40 also can effect the separation of serotypes of adeno-associated viruses and of adenovirus serotypes (Zweig and Salzman).

In chemical studies we have been able to react the viral DNA with a chemical probe that only reacts with single stranded regions of nucleic acid. In this way it has been possible to confirm that covalently closed double stranded molecules have areas which react as single stranded regions, and it has been possible to map these regions in precise regions of the viral genome and determine the degree of single strandedness. Perhaps the most useful part of this study is to provide an approach to determine whether these single stranded regions have some unique function, such as sites where RNA transcription is initiated (Chen, Lebowitz, Salzman).

The procedures that were previously used to map SV40 transcripts has been extended to answer other important questions. In outline, SV40 DNA is fragmented by bacterial restriction enzymes into genetically discrete double stranded DNA pieces ranging in size from 720,000 to 74,000 daltons. Each of these fragments is obtained in a pure form by gel electrophoresis and then each double stranded fragment is denatured and the complementary strands are separated. Using SV40 DNA, and cleaving with an enzyme from H. influenza, which makes eleven breaks in the molecule, you finally obtain 22 pools of viral DNA. Each of these corresponds to a unique part of the viral genome and they represent powerful probes in studies to determine expression of the viral genome in both lytically infected and transformed cells. When SV40 transformed cell lines were examined, using these probes, some interesting new findings emerged. As was already well documented, the major species of viral messenger (m)RNA within these cells is similar to mRNA that is found early in a lytic cycle of replication. In addition, low levels of mRNA are detected which are complementary to late viral mRNA. The characterization of these species has provided information on the way in which viral DNA is integrated into the viral genome, and provides an approach to locating the site of initiation of viral mRNA synthesis when the template is integrated in the cell genome (Khoury, Martin). In many studies which relate to transcription of viral mRNA there is some uncertainty since two factors, regulation of transcription and post-transcriptional processing of transcripts both can regulate and determine the kinds of mRNA that exist within cells. The isolation of transcriptional intermediates may clarify the nature of the primary transcripts and studies are currently under way in which nascent molecules of viral RNA that are still bound to the viral DNA template are being characterized (Birkenmeier, Rosenthal, Salzman).

An interesting demonstration of the way in which molecular procedures can be used to understand new biologic phenomena is demonstrated in recent studies with a human papovavirus - the BK virus. A number of isolations of papovaviruses have been made from patients who are immunosuppressed and from patients with Wiskott-Aldrich Syndrome. Antibodies which react with the virus are present in 80% of the adult human sera that have been tested. Applying the elegant methods previously used to study SV40, it has been shown that the BK virus is similar to SV40 in only 20% of the viral genome, and the similarity is in the fraction of the DNA that codes for late functions. By the use of restriction enzymes precise genetic maps of BK virus have been constructed. Cells have been transformed with BK DNA and these transformed cell lines are being characterized (Martin, Khoury, Howley).

Studies of SV40 DNA synthesis have continued and have focused on two aspects of replication - termination and initiation of DNA synthesis. The termination site for DNA synthesis has been firmly located 0.5 genome lengths from the site for initiation of DNA synthesis. The termination product is a DNA II molecule which has a gap of about twenty nucleotides. The site for termination of DNA synthesis is not controlled by a specific nucleotide sequence but rather is determined by the meeting point of the two replication forks. Studies of initiation have involved attempts to isolate young replicating molecules and to define the mechanism of initiated (Chen, Birkenmeier, Salzman).

As we gain additional understanding of viral transformation, the picture that emerges is that the product of the early viral gene, the T-antigen, is necessary to establish and to maintain the transformed state. Studies are being carried out in this laboratory and elsewhere to obtain the T-antigen in a purified form in order to determine how it functions in the transformed cell (Barban).

Adenoviruses.

This past year the studies with adenovirus focused on the structure of the viral genome. In past years, it was shown that the double stranded linear DNA molecules could be denatured and on renaturation they would give rise to single stranded circles. The basis for circle formation is the presence of similar sequences of nucleotides at both ends of a strand of adenovirus DNA; however, one sequence is inverted relative to the other. Studies of adenovirus type 18, have provided direct visual evidence for arrangement of bases. The viral genome also contains tandem complementary sequences within the genome. These regions reanneal rapidly and can be shown to be clustered in specific parts of the molecule. The function of these unique structures within the DNA is not yet established (Garon, Rose).

Parvoviruses.

Previously, the Kilham Rat Virus was shown to have an enzyme associated with the purified virion. This enzyme has been purified to homogeneity and has properties which distinguish it from other DNA polymerases within the infected cells. It is of great interest that one of the three virion proteins also serves a second function as a DNA polymerase (L. Salzman).

Studies with a second parvovirus, adeno-associated virus (AAV), have focused on the defectiveness of this group, and the way in which adenoviruses functions as helper viruses; that is, cells must be coinfected with AAV and adenoviruses in order to allow AAV to replicate. Two adenovirus ts mutants which are unable to synthesize viral DNA synthesis and, therefore, which do not synthesis late viral m-RNA and viral coat proteins have been used in these studies. At the restrictive temperature both of these ts mutants function as helpers for AAV. Since they do not carry out late viral functions, this suggests that some early adenovirus function is required for AAV replication. This system, in which AAV replication occurs, and in which there is no synthesis of adenovirus DNA is ideal for studying AAV DNA replication. In this study concatamers have been identified that are made up of + and - strands of AAV DNA. These concatamers can be shown to be precursors of the single stranded DNA that is finally incorporated into virions (Straus, Sebring, Rose).

Poxviruses.

Studies of the broadest biologic impact have come out of work with vaccinia virus m-RNA. Initially, in studies of cell m-RNA by Perry and his co-workers (at the Cancer Research Institute in Philadelphia) it was noted that certain bases in cellular m-RNA were methylated, and it seemed possible that these methylated bases might have some special role either in regulating the processing of m-RNA or in its functioning. Moss and his co-workers initiated studies with poxviruses which contain enzymes for the synthesis of viral m-RNA, since, in contrast to cells, poxviruses provide a system in which a well defined population of messenger molecules are made. They found that viral m-RNA was synthesized and it was methylated. Subsequently, it was shown that the methylated base was at the terminal 5' end of messenger RNA and they were able to define the structure of a unique dinucleotide that was present at the end of the m-RNA; 7-methylguanosine is linked by a 5' to 5' triphosphate link to an O-methylated nucleoside. The enzymes involved in the modification of the 5' end of m-RNA have now been purified from virions. This peculiar terminal structure has now been found in the same 5' terminal position in SV40 and adenovirus mRNA as well as in cell mRNA. It has also been shown that until mRNA has this specific terminal sequence, it cannot bind to ribosomes and be translated. This unique structural feature of m-RNA appears to be of universal importance and is likely to provide new insight into regulation of transcription and translation (Moss, Wei, Ensinger, Martin).

In other studies with poxvirus DNA, restriction enzymes have been used to fragment the viral genome into discrete pieces. Two fragments contain DNA which reanneals rapidly and they represent the ends of molecule which are covalently linked (DeFilippes).

General Laboratory Affairs.

Four members of the laboratory, Drs. Khoury, Moss, Rose and Salzman have served as organizers of sessions at the International Congress of Virology. Drs. Khoury and Salzman will serve as session chairmen during the meeting.

Dr. Moss will chair a session at the Gordon Conference on "Animal Cells and Viruses." For the past several years the focus of the LBV has been studies with DNA viruses. Drs. Khoury, Moss, Rose and Salzman all were authors of chapters in Vol. II of Comprehensive Virology. This volume dealt with DNA viruses and is one in a series published by Plenum Press and edited by Fraenkel-Conrat and R. Wagner. Dr. Martin has been appointed to the Virology study section for a four-year term and Dr. Lois Salzman has served as chairwoman of the Clinical Research Committee. Dr. Moss continues to serve on the editorial boards of the Journal of Virology, Antibiotics and Chemotherapy and Intervirology. Dr. Salzman, who has served for nine years as editor of the Journal of Virology will give up this position, but will now join the editorial board of that journal. Dr. Salzman has also been appointed as professorial lecturer in the Department of Microbiology at Georgetown Medical School.

This summer we will lose some excellent research associates and staff fellows, Drs. Howley, Shih, Straus and Zweig, and we look forward to the arrival of two new research associates, Drs. Boone and Israel. Other new staff members will be Dr. Moshe Shani from the Weizmann Institute (visiting fellow), Dr. Franz Ferdinand from the University of Konstanz, Konstanz, West Germany (visiting fellow), Dr. Evelyne May from the Cancer Institute in Paris (visiting associate), Dr. Michael Seidman from the University of California in Berkeley (staff fellow), Dr. Mark Bueller from the University of Glasgow (visiting fellow), Dr. Hardy Chan from the University of Wisconsin (visiting fellow) and Dr. Marshall Bloom from the Rocky Mountain Laboratory of NIAID (staff fellow). With the arrival of these bright young scientists we look forward to an exciting new year when we hope to gain new insights into ongoing problems.

Serial No. Z01 AI 00121-01 LBV
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Expression of Viral DNA During Lytic and Transforming Infection

Previous Serial No.: None

Principal Investigators: Dr. George Khoury

Other Investigators: None

Cooperating Units: Dr. Malcolm Martin
Dr. Barrie Carter
Dr. Heiner Westphal
Dr. Carlo Croce, Wistar Institute, Philadelphia, Pa.
Dr. Daniel Nathans, Johns Hopkins School of Medicine, Baltimore, Md.
Dr. Paul Lebowitz, Yale University Medical School, New Haven, Conn.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	22/12			22/12
Professional:	12/12			12/12
Other:	10/12			10/12

Project Description:

In collaboration with Dr. Daniel Nathans we were able to map the virus specific RNA found in SV40 infected and transformed cells. The early region was represented in abundant class of RNA in all transformed lines. In addition, a set of "anti-late" RNA sequences were present at lower concentrations, suggesting a possibly mechanism for transcription of integrated viral DNA.

We found that the SV40 - specific RNA present in the cytoplasm of lytically infected cells was asymmetric and represented approximately 48% of the minus (early) strand and 52% of the plus (late) DNA strand. These results differ considerably from our findings with nuclear RNA (which apperars to be in large part symmetric). Such results suggest a post-transcriptional processing of viral-specific RNA sequences either prior to or during transport to the cytoplasm.

With Dr. Paul Lebowitz, studies of defective hybrid virus, E46$^+$, were undertaken. We were able to show that while the adenovirus - SV40 hybrid contained a piece of SV40 DNA equivalent to 75% of the SV40 genome, only 50% of the genetic information was present. This apparent discrepency was later shown to result from a tandem reiteration.

With Dr. Barrie Carter, a restriction enzyme and transcriptional map of AAV-2 has been obtained.

In collaboration with Dr. Heiner Westphal, we have analyzed the post-transcriptional block to adenvirus 2 replication in monkey cells and the level of enhancement by SV40.

These projects are directed toward an understanding of the level of control of expression of early and late viral m-RNA in a replicative cycle of DNA viruses.

Key Words:

transcriptional and post-transcriptional controls, nucleic acid hybridization, restriction enzyme analysis, RNA processing, genome mapping, DNA integration.

Publications:

Martin, M. A., Khoury, G., and Fareed, G. C.: Specific reiteration of viral DNA sequences in mammalian cells. Cold Spring Harbor Symposiums on Quantitative Biology 39: 129-136, 1974.

Khoury, G., Howley, P., Brown, M., and Martin, M. A.: The detection and quantitation of SV40 nucleic acid sequences using single stranded DNA probes. Cold Spring Harbor Symposium on Quantitative Biology: 39: 147-152, 1974.

Carter, B. J., and Khoury, G.: Specific cleavage of adenovirus-associated virus DNA by restriction endonuclease R•Eco RI. I. Characterization of cleavage products. Virology 63, 523-539, 1975.

Lebowitz, P. and Khoury, G.: The simian virus 40 DNA segment of the adenovirus 7-SV40 hybrid, E46$^+$, and its transcription during permissive infection of monkey kidney cells. J. Virol., 15, 1214-1222, 1975.

Khoury, G., Martin, M. A., Lee, T.N.H., and Nathans, D.: A transcriptional map of the SV40 genome in transformed cell lines. Virology, 63, 263-272, 1975.

Khoury, G. and Salzman, N. P., Replication and transformation by Papoviruses, in Cancer: A Comprehensive Treatise, Plenum Press, New York, 1974, in press.

Khoury, G., Howley, P., Nathans, D. and Martin, M. A.; Posttranscriptional selection of SV40-specific RNA. J. Virol. 15, 433-437, 1975.

Eron, L., Westphal, H. and Khoury, G., Posttranscriptional restriction to adenovirus Expression in monkey cells. J. Virol., 15, 1256-1262, 1975.

Serial No. Z01 AI 00122-01 LBV
1. Biology of Viruses
2. Physical Biochemistry
3. Bethesda, Md.

Project Title: Biochemical Characterization of Papovavirus Genomes in Lytically Infected and Transformed Mammalian Cells

Previous Serial No: NIAID-80A

Principal Investigator: Malcolm A. Martin

Other Investigators: Peter M. Howley
Daniel Simmons

Cooperating Units: Kenneth Takemoto
Laboratory of Viral Diseases
NIAID, NIH

Daniel Nathans
Dept. of Microbiology
Johns Hopkins University
School of Medicine

Man Years:

	NIAID Lab. Staff	Other Personnel Assigned to Project	Guest Workers	Total
Total:	84/12		12/12	94/12
Professional:	34/12		12/12	46/12
Other:	48/12			48/12

Project Description:

During the past year, considerable effort has been devoted to the characterization of human papovavirus BK DNA. Antibodies to this agent, which was, originally isolated from the urine of an immunosuppressed renal allograft recipient, are present in 80% of adult human sera tested. Using bacterial restriction endonucleases, we have constructed physical maps of BK viral DNA and the genomes of two additional human papovaviruses, isolated from the urine of patients with the Wiskott-Aldrich Syndrome. Nucleic acid hybridization studies have revealed that each of the human papovavirus DNAs share approximately 20% of their polynucleotide sequences with SV40 DNA. This region of homology has been localized to those DNA sequences which are expressed late in the SV40 lytic cycle.

In collaboration with Dr. Kenneth Takemoto, LVD, NIAID, we have transformed rat and hamster cells with BK viral DNA using the calcium chloride precipitation method. Cloned BK viral transformed hamster cells: 1) are tumorigenic in newborn hamsters; and 2) contain 2-5 copies of BK viral DNA per cell.

The purpose of these studies is to characterize a papovavirus which is ubiquitous in the human population and determine its oncogenic potential in suitable biologic systems. Our aims are: 1) to compare several biological and biochemical properties (e.g. organization of DNA sequences, pattern of gene expression, mode of integration into mammalian DNA) of BK virus and SV40; 2) to isolate those viral DNA sequences responsible for the initiation and maintenance of the transformed state; and 3) to define those factors which regulate the latency BK virus in humans.

Key Words: Papovavirus DNA, physical map, polynucleotide sequence homology, virus transformed cells and integrated viral DNA

Publications:

Fareed, G. C., Byrne, J. C., and Martin, M. A.: Triplication of a unique genetic segment in an SV40 like virus of human origin and evaluation of new viral genomes. J. Mol. Biol., 87: 275-288, 1974.

Khoury, G., Fareed, G. C., Berry, K., Martin, M. A., Lee, T. N. H., and Nathans, D.: Characterization of a rearrangement of viral DNA: Mapping of the circular SV40 like DNA containing a triplication of a specific one-third of the viral genome. J. Mol. Biol. 87: 289-301, 1974.

Shih, T., and Martin, M. A.: Chemical linkage of nucleic acids to neutral and phosphorylated cellulose powders and isolation of specific sequences by affinity chromatography. Biochemistry, 13: 3411-3418, 1974.

Khoury, G., Martin, M. A., Lee, T. N. H., and Nathans, D.: A transcriptional map of the SV40 genome in transformed cell lines. Virology, 63: 263-272, 1975.

Howley, P. M., Mullarkey, M. F., Takemoto, K. K., and Martin, M. A.: Characterization of human papovavirus BK DNA. J. Virol., 15: 173-181, 1975.

Khoury, G., Howley, P., Nathans, D., and Martin, M.: Post-transcriptional selection of simian virus 40 specific RNA. J. Virol. 15: 433-437.

Serial No. Z01 AI 00123-09 LBV
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
IndividuaL Project Report
July 1, 1974 through June 30, 1975

Project Title: Characterization and Biosynthesis of Viral Enzymes and Structural Proteins.

Previous Serial No.: NIAID-80G

Principal Investigators: Dr. Bernard Moss

Other Investigators: Dr. Marcia Ensinger
Dr. Scott Martin
Dr. Cha Mer Wei

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	60/12		24/12	84/12
Professional:	24/12		24/12	48/12
Other:	36/12			36/12

Objectives:

1. Identification, isolation and characterization of viral enzymes and structural proteins with the aim of elucidating their roles in viral replication.

2. Determination of regulatory mechanisms involved in the biosynthesis of viral macromolecules and control of host functions.

Methods:

Viruses are grown in cultured cells and purified by density gradient sedimentation. Enzymes and other radioactively labeled macromolecules are purified from viruses or infected cells by sedimentation, chromatography, and electrophoresis.

Major Findings:

1. Discovery of a novel sequence of modified nucleosides at the 5'- terminus of viral and eukaryotic mRNAs. In vaccinia mRNA the sequences are $m^7G(5')ppp$

(5') Gm and $m^7G(5')ppp(5')Am$. In HeLa cells the sequences are m^7 $G(5')ppp(5')Nm$ and $m^7G(5')ppp(5')NmpNm$ in which Nm is any of the four 2'-O-methylribonucleosides ribonucleosides. An additional difference between vaccinia and HeLa cell mRNA is the presence of m^6A residues in the latter RNA.

2. Isolation of enzymes in vaccinai virus cores that modify the 5'-terminus of mRNA. These enzymes have been identified as guanylyl and methyl transferases. The steps in the modification are

$$\text{GTP + ppN-} \quad \text{GpppN- + PPi}$$

$$\text{GpppN- + S-adenosylmethionine} \quad m^7\text{GpppN- + S-adenosylhomocysteine}$$

$$m^7\text{GpppN- + S-adenosylmethionine} \quad m^7\text{GpppNm- + S-adenosylhomocysteine}$$

Significance to Biomedical Research:

The discovery that the 5'-terminus of cell and virus mRNAs are modified is an extremely important and exciting finding. This modification may play a key role in the regulation of cell and viral protein synthesis.

Proposed Course:

1. Purify and characterize viral and cell enzymes responsible for the 5'-terminal modification.

2. Determine the function of the modification in protein synthesis and whether it plays a role in regulatory events.

Key Words:

Virus enzymes, poxviruses, messenger RNA, post-transcriptional modification, virus structural proteins, vaccinia virus, HeLa cells, anti-viral agents, modification of RNA, methylation.

Publications:

Moss, B.: Reproduction of poxviruses. In Fraenkel-Conrat, H. and Wagner R. R. (Ed.): Comprehensive Virology, Vol III. Plenum Press, New York, 1974, pp. 405-474.

Paoletti, E., Rosemond-Hornbeak, H., and Moss, B.: Two nucleic acid-dependent nucleoside triphosphate phosphohydrolases from vaccinia virus: purification and characterization. J. Biol. Chem., 249: 3273-3280, 1974.

Paoletti, E. and Moss, B.: Two nucleic acid-dependent nucleoside triphosphate phosphohydrolases from vaccinia virus: nucleotide substrate and polynucleotide cofactor specificities. J. Biol. Chem., 249: 3281-3286, 1974.

Rosemond-Hornbead, H., Paoletti, E., and Moss, B.: Single-stranded DNA specific nuclease from vaccinia virus: purification and characterization. J. - Biol. Chem., 249, 3287-3291, 1974.

Rosemond-Hornbeak, H. and Moss, B.,: Single-stranded DNA specific nuclease from vaccinia virus: Endonucleolytic and exonucleolytic activities. *J. Biol. Chem.*, 249: 3292-3296, 1974.

Paoletti, E., Cooper, N. and Moss, B.: Regulation of synthesis of two immunologically distint nucleic acid-dependent nucleoside triphosphate phosphohydrolases in vaccinia virus-infected HeLa cell. *J. Virol.* 14, 578-586, 1974.

Moss, B. and Rosenblum, E. N.: Vaccinia virus polyriboadenylate polymerase: covalent linkage of the product with polyribonucleotide and polydeoxyribonucleotide primers. *J. Virol.* 14: 86-98, 1974.

Wei, C. M. and Moss, B.: Methylation of newly synthesized viral messenger RNA by an enzyme in vaccinia virus. *Proc. Natl. Acad. Sci. U.S.A.* 71, 3014-3018, 1974.

Wei, C.M. and Moss, B.: Methylated nucleotides block 5'-terminus of vaccinia virus messenger RNA. *Proc. Natl. Acad. Sci. U.S.A.*, 72, 318-332, 1975.

Wei, C. M., Gershowitz, A. and Moss, B.: Methylated nucleotides block 5'-terminus of HeLa cell messenger RNA. *Cell*, 4, 379-386,, 1975.

Kleiman, J.H. and Moss, B.: Purification of a protein kinase and two phosphate acceptor proteins from vaccinia virions. *J. Biol. Chem.* 250, 2420-2429, 1975.

Kleiman, J. H. and Moss, B.: Characterization of a protein kinase and two phosphate acceptor proteins from vaccinia virions. *J. Biol. Chem.* 250, 2430-2437, 1975.

Moss, B., Rosenblum, E. N. and Gershowitz, A. : Characterization of a polyriboadenylate polymerase from vaccinia virions. *J. Biol. Chem.*, in press, 1975.

Ensinger, M. J., Martin, S. A., Paoletti, E. and Moss, B.: Modification of the 5'-terminus of mRNA by soluble guanylyl and methyl transferases from vaccinia virus. *Pro. Natl. Acad. Sci. U.S.A.*, in press, 1975.

Moss, B., Ensinger, M. J., Martin, S. A., Wei, C. M.: Modification of the 5'-terminus of mRNA by guanylyl and methyl transferases from vaccinia virus. In Beaud, G. and Haenni, A.-L. (Ed.): In vitro transcription and translation of viral genomes. INSERM-EMBO, Paris, in press, 1975.

Rosemond-Hornbeak, H. and Moss, B.: Inhibition of host protein synthesis by vaccinia virus: Fate of cell mRNA and synthesis of small poly(A)-rich polyribonucleotides in the presence of actinomycin D. J. Virol. 16, in press, 1975.

Serial No. Z01 AI 00124-06 LBV
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Replication of the Parvovirus, KRV in an Infected Cell

Previous Serial No.: NIAID-80H

Principal Investigators: Dr. Lois Salzman

Other Investigators: Ms. Louese McKerlie

Cooperating Units:

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	32/12			32/12
Professional:	12/12			12/12
Other:	20/12			20/12

Project Description:

The parvoviruses have been found associated with or implicated as the causative agent in many disease states including several types of malignancies, infections hepatitis, gastroenteritis. The prototype of the non-defective parvoviruses is the Kilham rat virus(KRV). I wished to study the replication of the deoxyribonucleic acid and proteins of this virus in an infected cell line and also the impact the virus infection has on the cell synthetic processes.

In the past year I have worked on the purification and characterization of a DNA polymerase enzyme found associated with the purified KRV virion. The DNA polymerases from the cell were also isolated purified and characterized for comparison with the viral associated enzyme. The viral-associated DNA polymerase was purified to apparent homogeneity. It differs from the cellular polymerases in chromatographic properties, salt requirements and substrate specificities. It is hoped that further characterization of the viral-associated enzyme including the time of its appearance in the infected cell may lead to a better understanding of virus replication and its dominance over cellular functions.

We have also studied the ribonucleic acid (RNA) in KRV infected cells. The

RNA studied was determined to be viral specific on the basis of hybridization and base ratio data. We have determined the time of appearance of the viral RNA and found that the predominate viral RNA has a molecular weight of 6.5 to 7.5 x 10^5 and would account for 40 to 50% of the viral genome. We plan further experiments with the viral RNA in order to map the virus DNA and to determine what transcribed viral specific proteins lead to cell control and lysis.

Key Words:

Viral Single-stranded DNA replication
Virus associated DNA polymerases
Viral transcription and translation
Nucleic acid hybridization
Chromatography of enzymes
Parvovirus (KRV)

Publications:

Salzman, L. A. and Redler, B.: Synthesis of Viral-Specific RNA in Cells Infected with the Parvovirus, Kilham Rat Virus. J. of Virol. 14: 434-440 1974

Salzman, L. A. and McKerlie, L.: Characterization of the Deoxyribonucleic Acid Polymerase Associated with Kilham Rat Virus. J. Biol. Chem. 250: 32-37, 1975

Salzman, L. A. and McKerlie, L.: Nuclear and Cytoplasmic Deoxyribonucleic Acid Polymerases from Rat Nephroma Cells. J. Biol. Chem. 250: 25-31, 1975

Salzman, L. A. Parvoviruses in Nayak, D. P. (Ed).: Molecular Biology of Viruses. New York, Marcel Dekker, Inc. in press.

Serial No. Z01 AI 00125-06 LBV
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanism of DNA Replication and Integration

Previous Serial No. : Same

Principal Investigators: Dr. Norman P. Salzman

Other Investigators: Dr. Edward Birkenmeier
Dr. Michael Chen
Dr. Martin Zweig
Dr. Leonard Rosenthal
Ms. Nanette Tahner
Ms. Marilyn Thoren

Cooperating Units:

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	84/12		9/12	93/12
Professional:	48/12		9/12	57/12
Other:	36/12			36/12

Project Description:

The goal has been to understand the mechanisms that are used for SV40 DNA replication and those processes involved in transformation of cells by oncogenic viruses and to understand why DNA replication and transformation are coupled processes. We also are studing mechanisms for regulation of viral macromolecular synthesis.

While there is a specific initiation site for SV40 DNA synthesis and the nucleotide sequence of that region has been derermined, the mechanism of DNA initiation is not understood. This aspect of DNA replication is particularly important since it seems that the same viral gene product is involved in initiation of DNA synthesis and cell transformation. We can now isolate populations of replicative intermediates which have just started to initiate DNA synthesis and characterization of these molecules may provide a partial answer concerning how DNA synthesis is initiated. Termination of DNA synthesis produces daughter molecules that contain gaps of about twenty nucleotides at the termination site in the newly synthesized strans. The displacement of the termination site from the unexpected location suggests the presence of structures in the SV40 template which may inhibit the movement of one of the replication forks. To study regulation of viral RNA synthesis, we have isolated transcriptional intermediates in which nascent RNA is still bound to DNA, and conditions that regulate their synthesis are under study. Methods by which temperature sensitive mutants of SV40 can be separated from wild type virions have been developed and have been shown to be generally useful in separating serotypes of adenoviruses and of adeno-associated viruses.

Key Words:

SV40 DNA synthesis, DNA initiation, DNA termination transcriptional intermediates electron microscopic hetero-duplex mapping of viral m-RNA gel electrophoresis of virions.

Publications:

Sebring, E. D., Garon, C. F., and Salzman, N. P.: Superhelix density of replicating SV40 DNA molecules. J. Mol. Biol. 90:371-379, 1974.

Fareed, G. C. and Salzman, N. P.: The mechanism of SV40 DNA replication. In Tumor Virus--Host Cell Interaction. New York, Plenum Press, 1975, pp. 13-19.

Salzman, N. P. and Khoury, G.: Reproduction of Papovaviruses. In H. Fraenkel-Conrat & R. R. Wagner (Ed.): Comprehensive Virology. New York, N. Y. & London, 1974, pp. 63-141.

Salzman, N. P., Lebowitz, J., Chen, M., Sebring, E., and Garon, C.: Properties of replicating SV40 DNA molecules and mapping unpaired regions in SV40 DNA I. Cold Spring Harbor Symposium, Vol. 39, 1974, pp. 209-218.

Chen, M. C. Y., Chang, K. S. S., and Salzman, N. P.: Studies of polyoma virus DNA: Cleavage map of the polyoma virus genome. J. Virol. 15:191-198, 1975.

Serial No. Z01 AI 00126-02 LBV
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Isolation and Characterization of Restriction Enzyme which Produce Unique Pieces of Viral DNA

Previous Serial No.: NIAID-85A

Principal Investigators: Dr. Frank DeFilippes

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	15/12			15/12
Professional:	12/12			12/12
Other:	3/12			3/12

Project Description:

Restriction endonucleases which cleave vaccinia DNA into specific fragments are used to obtain a map of the genome. The covalently-linked complementary (CLC) ends of this DNA provide reference points. End fragments are separable from interior sequences by hydroxyapatite fractionation after sheared DNA is denatured and neutralized. Digestion of CLC fragments by restriction enzyme Hpa I produces 3 major and 4 minor segments as seen by electrophoresis. The largest major segment is 3.5×10^6 daltons and has one CLC end. Another segment of 10^6 daltons has the second end. Digestion with restriction enzyme Hpa II shows 4 major bands by electrophoresis. The largest band, containing one CLC end is 2.5×10^6, with a second of 0.9×10^6, daltons containing the other end.

The covalent link is short since elevated temperatures enhance single-strand specific deoxyribonulease digestion.

Another restriction endonuclease cleaves vaccinia DNA into large segments, 2 being 15×10^6 and 4-6, 8-10 $\times 10^6$ daltons. Segments of 2-5 $\times 10^6$ contain the ends.

Analysis of vaccinia DNA should show differently regulated gene classes and the spacing in and between classes. Regulation factors for this large DNA can be compared with those of small oncogenic virus DNA. A new restriction enzyme which yields large specific DNA segments will be used for this analysis.

Key Words:

Restriction Enzyme, vaccinia DNA

Serial No. Z01 AI 00127-08 LBV
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Structure and Function of Genetic and Protein Components of Adeno-Associated Viruses (AAV)

Previous Serial No.: NIAID-87A

Principal Investigators: Dr. James Rose

Other Investigators:
Dr. Steven Straus
Dr. Edwin Sebring
Dr. Frank Koczot
Dr. Claude Garon
Mr. James Garrison

Cooperating Units:

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	75/12			75/12
Professional:	51/12			51/12
Other:	24/12			24/12

Project Description:

Main objectives in studing these defective human parvoviruses are to (i) define specific biochemical mechanisms used in synthesizing their DNA, RNA and proteins, (ii) identify and characterize the helper virus-mediated step(s) required for their replication and (iii) relate biochemical findings to normal and altered cellular processes as well as to potentials for selective interference with both AAV and helper virus (adenoviruses, herpesviruses) infection. Among methods used are nucleic acid hybridizations, analytical and preparative sucrose and CsCl sedimentations, molecular cleavage with restriction nucleases, gel electrophoresis and electron microscopy. Major findings have provided new insites into (i) how AAV DNA is synthesized and (ii) the nature of adenovirus helper activity. First, we have identified and characterized concatermeric intemediates in AAV DNA replication. These molecules are up to six times unit length and consist of covalently linked plus and minus DNA strands, strongly suggesting that AAV DNA synthesis proceeds from a terminal self-priming sequence. This represents the best evidence yet for the existence of such an initiation mechanism in vivo. Secondly, we have found that two Ad 5 DNA minus temperature sensitive mutants are efficient helpers of AAV multiplication at the restrictive temperature. This indicates that the necessary helper function(s) is expressed early in the adenovirus cycle, and that AAV does not require all functions directly needed for adenvirus DNA synthesis. The molecular biology of AAV is revelant to understanding (i) natural modes of viral infection, (ii) biochemical processes in normal cells and (iii) possible approaches to the treatment of viral disease. We propose to continue studies of AAV replication and defectiveness.

Key Words:

nucleic and hybridization, analytical and preparative sedimentation, restriction nuclease, electron microscopy, gel electrophoresis, defective parvoviruses, adenoviruses, herpesviruses, temperature sensitive mutants, self-priming, DNA inititition, concatermeric DNA, helper functions.

Serial No. Z01 AI 00128-08 LBV
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1,1974 through June 30, 1975

Project Title: Properties of Adenovirus DNA

Previous Serial No.: NIAID-87B

Principal Investigators: Dr. Claude F. Garon
Dr. James A. Rose

Other Investigators: Mr. Ronald P. Parr
Mr. James W. Garrison

Cooperating Units:

Man Years:

	NIAID Lab. Staff	Other Personnel Assigned to Project	Guest Workers	Total
Total:	28/12			28/12
Professional:	12/12			12/12
Other:	16/12			16/12

Project Description:

The major objective has been to characterize the molecular structure of human adenovirus genomes and to correlate these features with specific biochemical functions. This information should enhance our understanding of both lytic and latent interactions between this group of viruses and the cells they infect. Methods used include electron microscopic visualization of macromolecules, sucrose gradient sedimentation, agarose gel electrophoresis,restriction endonuclease cleavage mapping, and hydroxy apatite column chromatography. Major findings include: I) The first decisive definition of the molecular arrangement of sequences within the inverted terminal repetition of an adenovirus DNA. This was based on direct, visualization of the repetitious sequences in oncogenic Ad 18 DNA. These sequences are responsible for the closure of single-stranded circular DNA molecules by a duplex, antiparallel, hydrogen-bonded projection. II) Identification and partial characterization of multiple sets of tandemly arranged, complementary sequences. The sequences are distinct from the inverted terminal repetition found within the genome of Ad 18 and are clustered in a specific portion of the genome, based on restriction endonuclease fragmentation studies. They can be isolated from other portions of the viral DNA as a result of their extremely rapid reannealing properties.

These sequences may represent important control elements in the expression of the adenovirus genome. The ability to obtain both physically and genetically defined DNA segments is an important prerequisite to the precise mapping of functions involved in viral replication or carcinogenesis or both. We propose to continue structural analyses of the DNA of both oncogenic and non-oncogenic human adenoviruses with the ultimate goal of relating specific regions of the genome to biochemical activities which permit these viruses to influence or gain control of cellular functions.

Key Words:

Adenovirus, nucleic acid, electron microscopy, heteroduplex analysis, restriction endonuclease fragmentation, DNA structure, inverted terminal repetition, genetic relatedness.

Publications:

Bartok, K., Garon, C. F., Berry, K. W., Fraser, M. J., and J. A. Rose, 1974. Specific Fragmentation of Adenovirus Heteroduplex Molecules with Single-Strand Specific Nucleases of Neurospora Crassa. J. Mol. Biol. 87, 437-449.

Serial No. Z01 AI 00129-05 LBV
1. Biology of Viruses
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the Biosynthesis and Characterization of Proteins in SV40 Infected and Transformed Cells

Previous Serial No.: NIAID 88

Principal Investigator: Dr. Stanley Barban

Other Investigators:

Cooperating Units:

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	19/12			19/12
Professional:	12/12			12/12
Other:	7/12			7/12

Project Description:

The objectives of this study are to isolate and identify those proteins specified by the SV40 viral genome early in the infectious cycle and the antigens produced by the transforming event. Since the SV40 virus can code for only a limited number of proteins these studies are directed to eventually identifying all the gene products. One of the gene products may be involved in the initiation and maintenance of the transforming event. To this goal, we have partially purified from a SV40 transformed mouse cell line the early tumor antigen (T-antigen) by subcellular fractionation procedures, column and affinity chromatography. The isolated protein has been analyzed and characterized by immunological methods, gel electrophoresis, density gradient centrifugation and by assays measuring the interaction of the protein with viral and cellular DNA. A quick assay method has been developed employing nitrocellulose membranes for examining the binding of the partially purified tumor antigen to DNA. It has been found that the tumor antigen preferentially binds to double stranded SV40 viral DNA and to a lesser extent single stranded viral nucleic acid. We expect to further purify this protein and fully characterize the antigen and possibly learn about its biological role in virus transformation or cellular regulation.

Keywords: SV40 Virus-Proteins, SV40 Tumor Antigen, SV40 Protein-Nucleic Acid Interaction

Serial No. Z01 AI 00130-05 LBV
1. Biology of Viruses
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemisty of Chromatin and Nucleic Acids

Previous Serial No.: NIAID-88B

Principal Investigators: Dr. Thomas Y. Shih

Other Investigators: Dr. Malcolm Martin
Dr. George Khoury

Cooperating Units:

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	18/12			18/12
Professional:	14/12			14/12
Other:	4/12			4/12

Project Description:

This project is to study the chemistry and biology of nucleic acids and chromatin, their relationship to the regulation of viral gene expression during transforming and lytic infections. By empolying SV40 DNA fragments chemically attached to cellulose matrix with a water soluble carbodiimide, viral specific RNA has been isolated from SV40 infected BSC-1 cells late in the lytic cycle. Exhaustive hybridization to SV40 DNA indicated that nearly all of the isolated RNA molecules contained SV40 sequences. The purity was close to 100%. The major 16S viral RNA species was isolated largely intact as monitored by sedimentation through neutral sucrose. Purified viral specific RNA is a valuable reagent for use in experiments characterizing eukaryotic RNA structure, synthesis and metabolism. Such a RNA can also be used in suitable cell-free translating systems generating the specific protein gene products coded for by the viral genome. There are one to three copies of the SV40 genome integrated into the transformed cell DNA. By employing SV40 cRNA prepared *in vitro* and covalently linked to a phosphocellulose matrix, the integrated SV40 DNA sequences have been purified 1,000 to 10,000 fold from the DNA of a SV40 transformed cell line (11A8). The exact location of integration sites of the viral genome can be examined by EM heteroduplex mapping using the purified DNA framents. This project will be terminated.

Key Words:

nucleic acids, chromatin, gene regulation, SV40, affinity chromatography, RNA-DNA hybridization, carbodiimide, mRNA, viral integration, gene isolation,viral gene function, cell transformation.

Publications:

Shih, T. Y. and Martin, M. A.: chemical linkage of nucleic acids to neutral and phosphorylated cellulose powders and isolation of specific sequences by affinity chromatography. Biochemistry, 13, 3411-3417, 1974.

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LMI

LPD

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LABORATORY OF MICROBIAL IMMUNITY

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PHS-NIH
SUMMARY STATEMENT

ANNUAL REPORT OF THE LABORATORY OF MICROBIAL IMMUNITY, NIAID
July 1, 1974 - June 30, 1975

Richard Asofsky, M.D.
Chief, Laboratory of Microbial Immunity

Studies in this Laboratory concern primarily the basic mechanisms involved in cellular and humoral immunity to microbial and tissue antigens. A full range of microbial antigens are used, including several viruses, two blood parasites, and pneumococcal polysaccharides. Work on tissue antigens are confined largely to graft-versus-host (GVH) reactions. An attempt is made to use the newest findings in basic immunology in this experimental work. The Laboratory has in consequence made contributions to this basic knowledge, such as the identification of suppressor thymus-derived cells (T-cells) and the description of functional heterogeneity among T-cells. This year many new experimental pathogens of mice were worked with, in hope of gaining understanding of human disease. In addition, plasma cells forming two classes of immunoglobulin were found both in plasma cell tumors and in an amplified immune response; the central importance of T-cells in resistance to several infections was demonstrated; a new kind of T-cell was found responsible for certain GVH reactions; and work on suppressor T-cells suggested that they were generated early in life and that they appeared to act by regulating proliferation of antibody forming cells.

The Laboratory moved from its location in Building 8 to renovated quarters in Building 5. The new laboratories are more ample but the total space much less flexible. We now have a centralized instrument room, and Dr. Tigelaar has established a tissue culture room used by several investigators. Experiments were in full progress within a week of the move. All investigators are very satisfied with the new arrangements for space.

Two Research Associates joined the Laboratory in July, Dr. Fredric Weinbaum and Dr. Henry Herrod. Dr. Jeffrey Jones left for a Residency in medicine after two very productive years. Dr. Vilho Pasanen, a Visiting Fellow left to do research at the Sloan-Kettering Institute when his appointment expired. Dr. Mirdza Neiders joined the Laboratory as a Guest Worker, first on sabbatical from The State University of New York at Buffalo, then under an IPA agreement, extended through 1976.

PROPERTIES OF PLASMA CELLS-DOUBLE IMMUNOGLOBULIN SYNTHESIS.

It is an accepted tenet of current immunologic theory that lymphocytes become plasma cells through separate lines of differentiation, such that each plasma cell synthesizes only one class of immunoglobulin. This was disproved in two independent experiments:

1. Seven percent of BALB/c mice bearing primary plasma cell tumors were found to have more than one paraprotein in their ascites. Many of these

appeared to be instances of two tumors arising independently. In two cases however, tumors designated TEPC 609 and SAMM 368, serial transplantation of over 20 generations of each also gave ascites or serum containing the same 2 immunoglobulin classes of paraprotein as the original ascites (IgA and γ2b in both instances; TEPC 609 have λ light chains, and SAMM 368 proteins have κ light chains). Tumor cells in both cases, but not from other plasma cell tumors, produced both products in vitro. Staining with fluorescein-labeled anti γ2 and rhodamine-labeled anti IgA, or with antibodies reciprocally labeled with these fluorochromes showed both red and green fluorescence in almost every cell in both tumors. The fluorescent reagents stained other plasma cell tumors singly. (Drs. Morse, Asofsky, LMI; Dr. Potter and Miss Pumphrey, NCI, and Dr. Lawton, University of Alabama).

2. BALB/c x C57 B16F_1 mice produce a very augmented antibody response to the pneumococcal polysaccharide SSS-III if they are given BALB/c T-cells at the time of immunization. The response was measured using sheep cells coated with SSS-III as indicators in a localized hemolysis in gel technique. Placques (PFC) developed with complement only were synthesizing IGM only, and could be blocked by mixing anti-IgM with the indicator cells. IgG antibody forming cells were identified by adding anti γ1 or anti γ2 and fresh complement, and counting additional placques (sequential method). When this was done after direct placques were developed, few additional placques appeared. However, if direct placques were blocked, many additional placques were seen (blocked method). Sequential and blocked methods gave identical numbers for IgG PFC in antibody responses to sheep erythrocytes, DNP-ficoll, and NNP-BSA. It was concluded that in the instance of SSS-III antibody formation, single cells made both IgM and IgG antibodies. Supporting this view were the observations that the "doubling" was not seen before day 5 nor after day 6, and that IgA PFC, developed with specific anti IgA were numerically identical no matter which method was used. (Drs. Pasanen, Baker and Asofsky).

[illegible]

1. Virus infection. Mice are naturally resistant to infection with herpes simplex hominis. Extensive depletion of T-cells by thymectomy, irradiation, bone marrow reconstitution, and treatment with antithymocyte serum (ATxBM-ATS) resulted in very increased susceptibility to lethal infection. Such depleted mice could be protected by high titer antibody, but also by infusion of normal spleen cells or even better by immune cells. Animals rendered incapable of making antibody and depleted of B-lymphocytes by treatment from birth with anti IgM proved very resistant to this infection, and their cells protected ATxBM-ATS syngeneic mice very well. (Dr. Morse, LMI and Dr. Burns, NIDR).

2. Parasite infection. Congenitally athymic mice were very susceptible to infection with a nonlethal varient of the mouse malaria Plasmodium berghei yoelii. Such mice could be protected almost completely with thymic grafts. AtxBM-ATS mice were also susceptible, and could be similarly protected with thymic graft. An in vitro assay of T-cell proliferation was developed to follow the course of immunity. Significant stimulation was found as early as 2 days after infection, and rose to very high levels where it was maintained

for many months. Reactivity of cells could be destroyed by antibody to the T-cell specific antigen Thy-1-2 and complement. (Drs. Weinbaum and Tigelaar, Mr. Evans).

In other work delayed hypersensitivity (DH) was also found early after infection or immunization with killed parasites. This DH could be augmented greatly by pretreating animals with cyclophosphamide. (Dr. Finerty).

In all cases, although T-cell immunity is clearly demonstrated, is related to protection, and some of its parameters can be measured quantitatively, the mode of action of this immunity remains unclear.

SUPPRESSOR T CELL ACTIVITY PRESENT EARLY IN LIFE.

Mice of various ages were evaluated with respect to (a) their ability to respond to an optimally immunogenic dose of Type III pneumococcal polysaccharide (SSS-III), (b) suppressor T cell activity, and (c) amplifier T cell activity. The ability to respond to SSS-III increased rapidly with age, reaching adult levels at 4 weeks of age; the response of 2 and 3 week old mice with only 7% and 40%, respectively, of that found in adult (8 week old) mice. Suppressor T activity was demonstrable at least as early as 2 weeks of age, and was just as profound as that noted in adult mice. However, amplifier T cell activity developed much more slowly and did not attain adult levels until 8-10 weeks of age. The dominance of suppressor T cell activity in young mice suggests that their inhibitory effects may contribute significantly to the ease with which tolerance is induced in neonates and help to prevent the development of auto-immune responses. (Drs. Baker, Morse, Prescott, Mr. Stashak and Ms. Amsbaugh).

SUPPRESSOR T CELLS REGULATE PROLIFERATION OF ANTIBODY-FORMING CELLS.

Mice immunized with an optimally immunogenic dose of Type III pneumococcal polysaccharide (SSS-III) were assayed at 2 hour intervals for the presence of antibody-producing plaque-forming cells (PFC); the kinetic data obtained were compared to those for mice, depleted of suppressor T cells by means of treatment with antilymphocyte serum. In both cases, the exponential increase in PFC proceeded in a stepwise fashion, and involved multiple nonrandom recruitment from a precursor cell population; however, additional stepwise increases in PFC occured in mice depleted of suppressor T cells, resulting in a 20-fold increase in the magnitude of the PFC response. These results, in conjunction with the findings of other studies, firmly establish that suppressor T cells act primarily by limiting the extent to which antibody-forming cells proliferate following immunization. (Drs. Jones, Baker, Prescott, Mr. Stashak and Ms. Amsbaugh).

NEW SUBLINE OF T-CELLS IDENTIFIED IN GVH REACTIONS.

F_1 mice show greatly augmented antibody responses to SSS-III when the antigen is given with parental cells (the allogeneic effect). Experiments were designed to identify the cellular component responsible for this manifestation of the GVH reaction. Donor tissues were compared to each other, and tissues from mice treated with sufficient ATS to eliminate GVH reactions of other kinds were compared to their normal counterparts. The findings were as follows: (1) thymocytes were active in GVH reactions but inactive in producing an allogeneic effect; (2) mixtures of lymph node (LN) cells and thymocytes, which produced enhanced GVH reactions showed the allogeneic effect expected from LN cells alone; (3) LN cells produced a somewhat greater allogeneic effect than blood lymphocytes; the reverse was true for GVH reactions; (4) ATS treatment greatly depleted (>90%) GVH activity of LN and spleen cells; the allogeneic effect was not depleted in spleen cells, and was not more than 50% depleted in LN cells; (5) T cells produce the allogeneic effect since this activity is destroyed by treatment of donor cells by anti Thy-1-2 and complement. (Drs. Pasanen, Baker and Asofsky).

Project No. Z01 AI 00133-09 LMI
1. Microbial Immunity
2. Microbiology & Immunity
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Molecular configuration in immunogenicity, tolerogenicity and the allergic response

Previous Serial Number: 113E

Principal Investigator: Philip R. B. McMaster

Other Investigators: James D. Owens, Richard Asofsky, David Rosenstreich*, William E. Paul**, John F. Finerty, Wilton Vannier***, Felix Rapaport****, Radoslav Bachvaroff****, Cornelius Glaudemons*****.

Cooperating Units:

*National Institute of Dental Research, NIH
**Laboratory of Immunology, NIAID, NIH
***Naval Medical Research Unit, Bethesda, Maryland
****Dept. Surgery, New York Univ. Med. School, New York City
*****Laboratory of Chemistry, NIAMDD, NIH

Man Years:

Total:	24/12
Professional:	12/12
Others:	12/12

Project Description:

The relation of antigenic size and shape to immunogenicity, tolerogenicity and the capacity to evoke an allergic response has been studied, particularly using substances that activate "bone marrow derived" B cells such as: Type III pneumococcal polysaccharide, dextran and Ficoll, and their conjugates with haptens.

The Type III pneumococcal polysaccharide showed an increase in immunogenicity when the size exceeded a threshold level of 13,000 daltons. When changed into an insoluble product, it could induce tolerance in mice and guinea pigs.

Coupling haptens to these bone marrow stimulating carrier molecules showed: the size of the carrier and hapten concentration both were important in the immunogenic and tolerogenic signals. As agents that can evoke an allergic response, they served to show that they can elicit hapten specific delayed responses as well as immediate reactions. Preliminary evidence shows that they may do that by exciting B cells rather than T cells.

The studies on molecular size indicate that by making an antigen large and insoluble, it can safely be used to tolerize guinea pigs without any risk of death from anaphylaxis. The possibility that this can be extended to other substances needs investigation. Its therapeutic implications are great

The work with haptens on B cell stimulators shows that the molecular siz and hapten density both are important in the immunogenicity and tolerogenicity of such compounds and in their ability to evoke an immune response. It also shows that more than one cell type may be important in delayed reactions and cellular type immune responses, and further suggests they may play a role in cellular immunity.

Keyword Descriptors:

- Antigen
- Cellular immunity
- Tolerance
- Pneumococcal polysaccharide
- Haptens
- Carbohydrates
- B-cells
- T-cells

Publications: None

Project No. Z01 AI 00134-13 LMI
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Control of immunoglobulin synthesis in mice.

Previous Serial Number: 116

Principal Investigator: Richard Asofsky

Other Investigators: Herbert C. Morse III, Pamela J. Sharp, Vilho Pasanen, Mirdza E. Neiders, William C. Paul*, Michael Potter**, Janet G. Pumphrey**, Alexander R. Lawton III***.

Cooperating Units:
*Laboratory of Immunology, NIAID
**Laboratory of Cell Biology, NCI
***Department of Pediatrics, University of Alabama, Medical Center, Birmingham, Alabama

Man Years:

Total:	36/12
Professional:	12/12
Others:	24/12

Project Description:

The purpose of these experiments is to gain insight into the factors governing the differentiation of plasma cells and antibody producing cells from their ancestor B-lymphocytes. Particular attention is paid to the differences between events or steps in this differentiation which are independent of antigenic stimulation and those which depend on such stimulation. Some work is done on differentiation of T-lymphocytes (derived from the thymus) and macrophages since interaction with the cell types seems required in many normal antibody responses.

Experiments this year were devoted primarily to the development of techniques for the separation of various cell types from each other. Polyvalent antimouse immunoglobulins were specifically purified by affinity chromatography on sepharose covalently coupled to purified mouse myeloma proteins. Elution was accomplished with weak acid (0.1M acetic). The purified, neutralized anti-immunoglobulins were then covalently coupled to sized sepharose beads (75-100µ) after the method of Schlossman. Columns made from such an absorbent can remove >90% of B-lymphocytes, as judged by staining with polyvalent anti-immunoglobulin or class specific anti IgM conjugated with fluorescin (IgM is a major immunoglobulin component on B-lymphocytes). Such Ig+ cells could be recovered almost completely by incubation of the column with mouse serum or isolated mouse myeloma proteins. The cells not absorbed on the columns contained most ($\sim$90%) of the T lymphocytes as judged by indirect fluorescence with anti Thy 1.2 followed by either fluorescent anti $\gamma 2$ or fluorescent anti IgM. Total yields from these columns was usually >90%.

In functional studies it was shown that the nonadherent pool of cells contained almost all cells responding to mitogens which stimulate T-lymphocytes (PHA and Con A) but reacted poorly with mitogens which stimulate B-lymphocytes (lipopolysaccharide; LPS). Ig+ cells eluted from the column did not respond to Con A or PHA but did respond to LPS. These results were obtained using a tube culture technique and minimum medium. In a microculture method using highly enriched media, responses to LPS were obtained in the nonadherent pool. Experiments examining the distribution of antibody forming cells and effector T cells (cytotoxic lymphocytes) are intended.

One polyvalent reagent, and Fab antibody, was found to stain more cells than are usually counted as B-lymphocytes, and to remove more cells in the above technique, especially when lymph node cells are used. If this is not an artifact then this reagent either detects immunoglobulin on T-lymphocytes, detects immunoglobulin on B-lymphocytes which other reagents fail to detect (perhaps cells early in differentiation) or both. Functional studies using this anti-Fab are planned.

Two plasma cell tumors which each produce two myeloma proteins (see: Z01-A1-00135-01 LMI) have been established in long term culture, and retain their characteristic double production. Cloning experiments are planned.

Past work has shown normal ontogenetic development of B-lymphocytes in germfree mice. This year appearance of T cells with time was examined and was found to be normal. Experiments are planned which will investigate functional development of the two lines of lymphocytes, using transfer of cells to irradiated recipients. This work will be combined with antiglobulin column fractionation.

Keyword Descriptors:

B-lymphocytes
T-lymphocytes
Plasma cells
Plasma cell tumors
Immunoglobulins
Anti-immunoglobulins
Fluorescence

Publications: None

Project No. Z01 AI 00135-01 LMI
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Properties of immunoglobulin secreting cells

Previous Serial Number: None

Principal Investigator: Herbert C. Morse III

Other Investigators: Richard Asofsky, *Michael Potter, ** Alexander R. Lawton III, **John Kierney, *Janet G. Pumphrey, Vilho Pasanen

Cooperating Units: *Laboratory of Biology, NCI, NIH
**Dept. of Pediatrics, Univ. of Alabama Medical Center, Birmingham, Alabama

Man Years:

Total:	14/12
Professional:	8/12
Others:	6/12

Project Description:

These investigations are designed to evaluate the functional capacities of both normal and malignant antibody forming cells during their development and in their full maturity.

A study of 778 consecutive primary plasmacytomas revealed that two or more paraproteins could be detected by immunoelectrophoretic analysis in the ascites of 7% of tumor bearing mice. In most cases, this finding probably represents the simultaneous occurrence of independently developing tumors in the same mouse, but two methods demonstrated that, at least in some instances, a single tumor clone is capable of producing two immunoglobulin classes. First, two tumors carried for 16 or more generations in transplantation produced two paraproteins in multiple generations. Second, double staining of individual plasma cells with rhodamine- and fluorescein-labeled class-specific antiglobulins revealed both secreted classes of immunoglobulin in single cells. For one tumor, TEPC 609, paraproteins of the $\gamma 2b$ and γA immunoglobulin classes were purified from ascitic fluid and shown to share idiotypic specificities.

Other studies suggested that the capacity to simultaneously express two immunoglobulin classes is a property of normal plasma cells as well as plasmacytomas. An allogeneic effect was produced in (BALB/c x C57BL/6)F_1 mice by intravenous administration of BALB/c spleen cells and intraperitoneal immunization with Type III pneumococcal polysaccharide (S3). Two to seven days later, injected mice were sacrificed and their spleens were analyzed for 1) the class distribution of S3-specific plaque-forming cells (PFC) and 2) the appearance of plasma cells containing more than one immunoglobulin class utilizing immunofluorescent analysis. Evaluation of the PFC response suggested that (5-6 days after allogeneic stimulation) a high proportion of PFC secreting γM antibody were also secreting either γ1 or γ2 S3-specific antibody. Fluorescence analysis of plasma cells from these mice revealed many cells containing more than one immunoglobulin class with all possible combinations of "doubles" of γ1, γ2, γA and γM classes detected.

These parallel studies in plasmacytomas and normal plasma cells provide unique models for studying the maturation of the humoral immune response; in particular, the mechanism(s) by which the "switch" from production of γM to production of other immunoglobulin classes proceeds.

Further work is planned to evaluate normal plasma cells for 1) their potential to secrete more than 2 immunoglobulin classes simultaneously; and 2) to determine if the rule of "allelic exclusion" is followed in plasma cells secreting more than one immunoglobulin class.

Analysis of plasmacytomas will be continued in a search for 1) immunoglobulin classes corresponding to IgD or IgE in man; 2) "double-producing" tumors secreting other class combinations; and 3) mechanisms by which these tumors might be caused to switch from production of one class of immunoglobuli to another.

Keyword Descriptors:

Plasma cells
Allogeneic effect
Immunoglobulin synthesis
Plasmacytomas
Immunoglobulin class switch

Publications: None

Project No. Z01 AI 00136-03 LMI
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Functional activities of subpopulations of thymus-derived cells

Previous Serial Number: 118B

Principal Investigator: Robert E. Tigelaar

Other Investigators: Richard Asofsky, Donald Mosier, Charles B. Evans, NIAID
*R. M. Gorczynski

Cooperating Units: *Ontario Cancer Institute, Toronto, Ontario, Canada

Man Years:

Total: 4/12
Professional: 4/12
Others: 0/12

Project Description:

Project Subtitle I: Regulation of the in vitro antibody response to pneumococcal polysaccharide

The purposes of the present studies have been to define the conditions necessary to obtain a satisfactory in vitro model for immunity to pneumococcal polysaccharide (SIII), to analyze the cellular requirements for this response, and to characterize the cells and/or factors which can augment or suppress that response.

The regulatory mechanisms involved in controlling the in vivo immune response of mice to Type III pneumococcal polysaccharide (SIII) have been the focus of a great deal of interest and study in recent years. These studies have strongly suggested that while the humoral antibody response to this antigen does not involve an obligatory cooperative interaction between thymus-derived (T) helper cells and bone marrow-derived precursors of antibody forming cells (B cells), T cells nevertheless can both amplify and suppress the activity of SIII-specific B cells. Further evidence suggests that suppressor T cells play an active role in the development of an unresponsive state (tolerance) to this antigen. (See also NIAID - 119A, 1971-1974.) As impressive as the sum of the available evidence is in support of T regulator cells in this in vivo model, alternative explanations for these findings do exist. A more definitive explication of the cellular requirements for the immune response to SIII, and the nature and mode of action of any cells which regulate this response requires either an in vivo adoptive transfer model or an *in vitro* model, so that the cell populations under question can be quantitatively manipulated and more adequately characterized. Previous attempts to obtain either of these two models has met with either no or only questionable success.

Mouse spleen cell suspensions obtained from either normal animals or animals previously primed with a low dose of SIII were cultured *in vitro* either separately or in appropriate mixtures with various antigens, utilizing the standard Mishell-Dutton culture system. Plaque-forming cells (PFCs) secreting antibody to these antigens were enumerated after varying periods of culture utilizing a modified hemolysis-in-gel assay. The cellular requirements of these responses were studied by determining the effects of pretreatment of the cells by adherence to plastic, passage through Sephadex G-10 columns, X-irradiation, or treatment with a variety of agents with affinity for cell membrane components (such as anti-thymocyte serum, anti-theta, anti-TL, anti-Ly, cortisone acetate, etc.). In addition, athymic nude mice were utilized as a source of cells devoid of T cells, and mice treated from birth with heterologous anti-immunoglobulin serum were used as a source of cells lacking functional B cells.

Significant reproducible IgM and IgG anti-SIII PFC responses have been obtained from normal BALB/c spleen cell suspensions cultured with small amounts of SIII in Mishell-Dutton cultures. Dose-response titrations indicated 5 x 10^{-} micrograms SIII/culture to be the optimally immunogenic concentration. Peak IgM PFC responses occurred after 4 days of culture, while the IgG PFC response peaked on day 5. Spleen cells from mice primed 48-72 hours before sacrifice with a low dose of SIII (0.005 micrograms) were also cultured for varying periods of time with various concentrations of SIII. The kinetics of both IgM and IgG responses of these primed cells was identical to that of unprimed cells. However, these primed cells, while giving equivalent PFC responses (compared to normal cells) to supraoptimal amounts of SIII, gave significantly suppressed responses (approximately 25% of normal) to the optimal or suboptimal doses of SIII. This suppression was antigen-specific: responses of SIII-primed cells to the unrelated antigens sheep erythrocytes and DNP-Ficoll, in the presence or absence of additional SIII in the cultures, were similar to that of normal cells. These *in vitro* findings confirm the previous *in vivo* observations that mice primed with a low dose of SIII give severely impaired PFC responses when challenged with an optimally immunogenic amount of SIII.

When small numbers (as few as 5%) of SIII-primed spleen cells were cultured together with normal cells in the presence of the optimal dose (for normal cells) of SIII, specific suppression of the anti-SIII PFC response resulted, while responses to sheep erythrocytes and DNP-Ficoll were unimpaired. Serum obtained from low dose SIII-primed animals was also found to markedly inhibit the response of normal cells to doses of SIII of 5 x 10^{-6} micrograms/culture or less; however, increasing the antigen dose overcame the inhibitory effects of this serum. Finally, an initial series of experiments was performed to see whether suppressor cells (obtained by low dose SIII priming) could be generated in animals treated from birth with heterologous anti-immunoglobulin serum, a treatment known to severely depress the development of most functional B cells. Small numbers of SIII-primed spleen cells from such mice, which lacked detectable numbers of Ig-bearing lymphocytes and produced to PFCs to

SIII, sheep erythrocytes, or DNP-Ficoll either before or after antigenic stimulation in vitro, could apparently specifically suppress the anti-SIII response of normal cells. These results would indirectly support the concept that low-dose tolerance to SIII may at least in part be mediated by an active suppressor cell which is not a B cell.

The proposed course of this project is to more definitively establish the nature of the suppressor cell present in spleens of low-dose primed mice. Further studies will be directed to the nature of the suppressive factor(s) found in the serum of such mice and the relationship of this serum-mediated suppression to the suppression observed with primed spleen cells. Finally, experiments will be directed toward the question of the target cell for and mode of action of these suppressor cells and factors. Such studies should facilitate a clearer understanding of some of the factors which regulate the expression of the immune response to an antigen whose biomedical significance is obvious.

Keyword Descriptors:

Thymus-derived (T) cells
Cell-mediated immunity
Plaque-forming cells
Bone marrow-derived (B) cells
Suppressor cells
In vitro immune response
Pneumococcal polysaccharide
SIII
Tolerance
Antibody response in vitro
Regulation of immune responses

Publications:

Gorczynski, R., Kontianen, S., Mitchison, N.A., and Tigelaar, R.E.: Antigen-antibody complexes as blocking factors on the T-lymphocyte surface. In Edelman, G. (Ed.): Cellular Selection and Regulation in the Immune Response. New York, Raven Press, 1974, pp. 143-154.

Tigelaar, R.E., and Gorczynski, R.M.: Separable populations of activated thymus-derived lymphocytes identified in two assays for cell-mediated immunity to murine tumor allografts. J. Exp. Med. 140: 267-289, 1974.

Gorczynski, R.M., and Tigelaar, R.E.: Cell-mediated immunity to murine tumor allografts. Increase in the activities of activated thymus-derived cells following in vitro incubation. Cell. Immunol. (in press).

Project No. Z01 AI 00137-09 LMI
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biology of graft-versus-host reactions.

Previous Serial Number: 116J and 118

Principal Investigators: Vilho J. Pasanen
Henry G. Herrod

Other Investigators: Richard Asofsky, Herbert C. Morse III, Phillip J. Baker

Man Years:

Total:	30/12
Professional:	24/12
Others:	6/12

Project Description:

The purpose of these studies is to investigate the cells which participate in, and the cell interactions which occur when allogeneic lymphoid cells are given to immunologically incompetent allogeneic recipients. Use is made os quantifiable and commensurate differences in activity either between tissues of different anatomic origin or differences after treatement of cell donors with such therapeutic agents as antithymocyte serum (ATS). Work in the past has established that two GVH activities can be measured by parallel line assays: (1) the capacity to induce splenomegaly, using the Simonsen spleen weight assay and (2) the capacity to produce lethal reactions (death) in lightly irradiated recipients. Usually, the activity of cells from BALB/c or C57Bl/6 mice are measured in (C57Bl x BALB/c)F$_1$ (BLCF$_1$) or BALB/c x C57B16)F (CBLF$_1$) newborn mice, as a function of the dose of parental cells. It has been established that certain combinations of cells such as thymocytes and lymph node cells produce much greater reactions than either population alone (synergy). It has consequently been deduced from this and other data that there must be functional heterogeneity among the T-lymphocytes which mediate the GVH reaction.

This year's work centered on a detailed analysis of the "allogeneic effect", thought to be a component of the GVH reaction. CBLF$_1$ mice were given intravenously graded doses of BALB/c cells from spleen, lymph node, thymus, peripheral blood; or from spleens or lymph nodes of mice treated previously with ATS in doses sufficient to suppress greatly GVH reactions as measured by both the spleen weight and mortality assays of GVH activity; or with combinations of such cells. At the same time, these recipients received an optimum immunizing dose of the pneumococcal polysaccharide SSS-III. Antibody responses were analyzed by counting the number of cells making specific antibody to SSS-III, using SSS-III coated sheep erythrocytes and a localized hemolysis in gel assay, at various days after immunization.

At the peak of the response (5-6 days) animals which received 10^7 - 8×10^7 parental cells showed a cell-dose dependent increase in the number of cells making antibody, which at a maximum was 25 fold or greater. Recipients of thymocytes showed no enhancement. Activities fell in the rank order LN>PBL>Spleen>Thymus=0. This is different from GVH reactions where PBL>LN> Spleen>Thymus which is active. No synergy was seen combining thymocytes with LN cells, as is seen in GVH reactions. Following treatment with ATS lymph node cells showed >90% suppression of GVH activity, but not more than a 50% decrease in capacity to produce an allogeneic effect. Spleen cells showed >90% decrease in GVH activity and no decrease in capacity to make an allogeneic effect. Immunization with C57Bl cells increased the GVH activity, but decreased the capacity to make an allogeneic effect. Cells treated with anti Thy-1-2 and complement were reduced >80% in their capacity to make an allogeneic effect. It was concluded that cells mediating the allogeneic effect were T cells belonging to a subset that was not measured by any previously used GVH reaction, and that the functional heterogeneity of T-cells must now include at least 3 separate sets of cells.

Another portion of the experiment sought to investigate "switching" from IgM to IgG antibody synthesis, a known concomitant of the allogeneic effect. At days 4 and 5 many cells were synthesizing both IgM and IgG antibodies (see Z01 AI 00135-01 LMI). At later times cells producing IgM and IgG antibodies belonged to different sets. There was never overlap between cells making IgM and those making IgA antibodies. Cells making two classes of antibody have been found as rare occurrences in antibody responses to sheep erythrocytes. This study shows that such double synthesis can be a part of the normal pathway of differentiation of B-lymphocytes to plasma cells.

Future work is intended to isolate the cells responsible for the allogeneic effect from those responsible for GVH reactions.

Keyword Descriptors:

Graft-versus-host reactions
Allogeneic effect
T-lymphocytes
B-lymphocytes
Plasma cells
Antibody synthesis
IgG
IgM
Pneumococcal polysaccharide
Antithymocyte serum

Publications: None

Project No. Z01 AI 00138-01 LM
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Viruses and the immune response

Previous Serial Number: None

Principal Investigator: Herbert C. Morse III

Other Investigators: ** Susan S. Cross, *Janet W. Hartley, ***William H. Burns, Philip J. Baker, Vilho Pasanen

Cooperating Units:
*LVD, NIAID, NIH
**Microbiologic Associates, Inc., Bethesda, Maryland
***LMI, NIDR, NIH

Man Years:

Total:	4/12
Professional:	4/12
Others:	0/12

Project Description:

The purpose of these studies is to evaluate the interaction of viruses and the immune system, both in terms of alterations produced by viruses in cell populations participating in the immune response as well as the immune response to viral antigens.

A murine herpes virus, mouse thymic virus, has been shown to produce necrosis of the thymus at 7-10 days of age when given to newborn mice, followed by gradual recovery of normal thymic structure. Spleen, thymus and lymph node cells from infected BALB/c mice were tested for their capacity to produce a graft-vs-host (GVH) reaction in neonatal F_1 recipients. A major deficiency in GVH-reactivity of thymocytes and spleen cells was demonstrated in mice 4-8 weeks after infection. Lymph node GVH reactivity was affected little if at all suggesting that the virus may selectively affect subpopulations of T-cells. This suggestion was supported by studies of the development of suppressor and amplifier T-cell populations which modify the response of B-cells to immunization with Type III pneumococcal polysaccharide (S3). Infected and normal mice 2-8 weeks of age were immunized with optimally immunogeneic doses of S3 and tested for enhancement of the plaque-forming cell (PFC) response by treatment with antelymphocyte serum as an indirect measure of amplifier T-cell function; no major differences between thymic virus-infected and normal mice could be detected at all ages tested. Suppres T-cell function was assessed indirectly by testing the capacity of low-dose

S3 priming to reduce the PFC response to an optimally immunogenic dose of S3; again, no differences between infected and normal mice of various ages could be detected suggesting that both suppressor and amplifier T-cell functions were relatively spared. These studies give further indications of heterogeneity among T-cell populations and suggest that they may be selectively altered by a T-cell-tropic herpes virus. These studies have been concluded.

Other work evaluated the immune mechanisms responsible for control of infection of mice with herpes virus hominus Type II. Mice extensively depleted of T-cell function by adult thymectomy, lethal irradiation, bone marrow reconstitution, and treatment with antithymocyte serum were much more susceptible to martality induced by herpes virus than normal mice. Partial protection from lethal infection of T-cell depleted mice could be conferred by injection of mice with 1) high titer immune serum; 2) high numbers of normal spleen cells; or 3) low numbers of immune spleen cells. The protective element in non-immune spleen cells appears to be due to T-cell function, perhaps through several mechanisms. Further studies are in progress to determine the role of T-cells in such protection through "helper" effects and macrophage activation.

Studies of the humoral immune response of mice to infection with endogenous xenotropic murine leukemia virus (X-MULV) have shown that congenitally athymic (nude) mice have serum neutralizing titers to the virus equivalent to that of their thymus-bearing littermates. These findings indicate that 1) mice are not tolerant to a virus expressed in their tissues from the time of birth; and 2) that the capacity to make neutralizing antibody is thymus-independent. Further work is planned to define the nature of the immunizing viral antigen and determine if T-cells can be shown to recognize the virus.

Keyword Descriptors:

Mouse thymic virus
Graft-vs-host reaction
Type III pneumococcal polysaccharide
Allogeneic effect
Lactic dehydrogenase virus
Xenotropic murine leukemia virus

Publications: None

Project No. Z01 AI 00139-10 LMI
1. Microbial Immunity
2. Microbiology & Immunolog
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The immunologic response of animals to trypanosomal antigens

Previous Serial Number: 117E

Principal Investigator: John F. Finerty

Other Investigators: Edward P. Krehl*

Cooperating Units: *Trudeau Institute, Saranac Lake, New York

Man Years:

Total:	6/12
Professional:	6/12
Others:	0/12

Project Description:

[illegible] was to investigate the role of cell-mediated immunity (CMI) on host protection to lethal Trypanosoma rhodesiense in mice. CMI was assayed by the delayed hypersensitivity reaction (DH) that occurred after immunization with T. rhodesiense antigens. DH, a function of T-cells, was measured by the injection of antigen, into the right-hand footpad (RHFP), equivalent to 10^6 trypanosomes, and the degree of footpad swelling measured 24 hrs later. Mice immunized with either formaledehyde treated (FO) or freez thawed (FT) antigens demonstrated DH reactions detectable 42 days after immun zation. The degree of DH was greater in animals immunized with $\geq 10^6$ antigen. Mice were immunized and challenged with 10^4 T. rhodesiense trypmastigates. Partial protection was demonstrated 2 days after immunization, but complete protection was observed 8 days after immunization. DH was observed in both protected and non-protected animals. These results indicate that DH can be induced to T-rhodesiense antigens, and is long lasting. Furthermore, animals can be protected against a lethal challenge by active immunization, but complete protection takes apparently 8 days to develop. Since DH was observec in protected and non-protected animals, this suggests: (1) 2 types of DH reactions were observed, or (2) different groups of antigens are involved in inducing protection vs those capable of merely inducing DH. The significance of this study is that protection against T. rhodesiense, a fatal disease in most mammals, can be induced in animals as soon as one week after immunizatio Future studies will focus on the type of DH observed in immunized animals, plus the role of cell-mediated immunity in conferring protection.

Keyword Descriptors:

Cell-mediated immunity
Delayed hypersensitivity
Trypanosoma rhodesiense

Publications: None

Project No. Z01 AI 00140-10 LMI
1. Microbial Immunity
2. Microbiology & Immunolog
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Immunology of malaria

Previous Serial Number: 117

Principal Investigator: John F. Finerty

Other Investigators: Edward P. Krehl*

Cooperating Units: *Trudeau Institute, Saranac Lake, New York

Man Years:

Total:	6/12
Professional:	6/12
Others:	0/12

Project Description:

It is the purpose of these studies to determine the nature of the host immune responses to the rodent malarial parasite Plasmodium berghei, and the role of these responses in host protection. Immune responses, particularly those of the cell-mediated immune system, were measured after immunization with antigens prepared from P. berghei or after infection induced by lethal and non-lethal strains of P. berghei. Cell-mediated responses were studied in mice using footpad swelling as an indicator of delayed-hypersensitivity reactions (DH) a function ascribed to T-cell response activity. Antigens used were of two types: (1) formaldehyde treated (FO) and (2) freeze-thawed antigens (FT). Delayed-hypersensitivity (DH) was elicited by injecting into the right-hind footpad (RHFP), FT antigen equivalent to 10^8 parasites in 50 μ the degree of footpad swelling was measured 24 hrs later. Mice were immunize with FO antigen, in concentrations of 10^3-10^8 killed parasites. Three routes of immunization used were intravenous (IV), subcutaneous (SC or the left-hind footpad (LHFP). DH measured 4 days after immunization revealed minimal response in animals immunized IV, and maximum by SC immunization. Then mice immunized SC with FO antigen and separate groups tested for DH on 4, 14, 21 and 42 days later. DH was detectable in all animals but was greater in animals immunized $>10^6$ FO antigen. Other groups of mice were then immunized SC, with either FO or FT antigens, and DH tested 14 days later with FO, FT or live P. berghei parasitized erythrocytes. Results of this study revealed an intense DH reaction to live parasites and demonstrated that immunization with killed parasites will sensitize mice to antigen found on live parasites. Mice were treated with cyclophosphamide (Cy) in order to enhance their DH response. Subsequently, these mice were inoculated intraperitoneally with 10^4 parasit erythrocytes; a non-Cy group was inoculated at the same time. DH was measured 2, 4, 6, and 8 days after infection. The Cy group demonstrated an intense DH 2 days after infection which decreased sharply on day 4, and remained at low levels through day 8. However, the non-Cy grouped showed only a slight DH response. Furthermore, the Cy group cleared their parasitemia and survived; the non-Cy group showed a rapid rise in parasitemia and succumbed as a result of infection. These results are significant for they demonstrate that DH, a function of T-cells, can be induced to malarial antigens, that the response is long lasting and sensitized animals can elicit a response to live malarial parasites. In addition, an enhanced T-cell response to lethal _P. berghei_ has a beneficial effect on host survival. Future studies will investigate other parameters of T-cell responses and their role in host protection against malaria.

Keyword Descriptors:

Cell-mediated immunity
Delayed hypersensitivity
Cyclophosphamide
Plasmodium berghei
Protection

Publications: None

Project No. Z01 AI 00141-01 LMI
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: T-cell sensitization in murine malaria

Previous Serial Number: None

Principal Investigator: Fredric I. Weinbaum

Other Investigators: Robert E. Tigelaar and Charles B. Evans, NIAID

Cooperating Units: None

Man Years:

Total:	32/12
Professional:	20/12
Others:	12/12

Project Description:

The purpose of these investigations has been to evaluate the significance of thymus-derived (T) cell sensitization in resistance to *Plasmodium berghei* in mice. Two models of T-cell immunodeficiency were used to confirm the thymic dependence of resistance to malaria in mice and to provide models for reconstitution studies to determine the cell populations involved: 1) adult thymectomized, X-irradiated, bone marrow reconstituted, and antithymocyte serum treated (ATx-BM-ATS)(BALB/c x C57 Bl/6) F_1 mice, and 2) congenitally athymic (nude) mice, NIH random bred Swiss background, and third backcross generation BALB/c background. Some of the BALB/c nude mice were grafted subcutaneously with neonatal BALB/c thymus grafts to reconstitute T-cell function. Serum immunoglobulin levels of IgM, IgG_1, and IgG_2 were measured by radial immunodiffusion. Antimalarial antibody was measured using indirect hemaglutination of fixed sheep erythrocytes tanned with a crude antigen preparation made from the supernate of freeze-thawed 17X lethal (see below) schizonts isolated on a Ficoll gradient.

Two strains of *P. berghei* were utilized: 1) 17X lethal (17XL) caused a fulminating, almost invariably lethal infection in BALB/c, $BLCF_1$, and NIH mice, and 2) 17X nonlethal (17XNL) caused a self-limited infection in these same strains. Following infection with 17XNL, mice were subsequently resistant to the lethal variant, 17XL. Spleen cells from BALB/c mice initially infected with 17XNL and then challenged with 17XL were used to define the characteristics of an *in vitro* proliferative response to *P. berghei* infected syngeneic RBCs and the previously mentioned crude antigen preparation.

The major findings of these investigations have been:

a. ATX-BM-ATS BLCF$_1$ mice were more susceptible than age- and sex-matched controls to both 17XL and 17XNL as determined by an increase in mortality and decrease in survival time. The difference seen with 17XL increased with decreasing parasite dose.

b. NIH nude mice succumbed (35/35) to infection with 17XNL while the phenotypically normal littermate controls survived (35/36). Third generation backcross BALB/c nude mice also succumbed (11/11) to 17XNL while the vast majority of thymus grafted BALB/c nude mice survived (11/13). Measurements of serum immunoglobulin levels of thymus-grafted versus sham-grafted BALB/c nudes were made prior to infection and 11 days after infection. IgG$_1$ and IgG$_2$ levels were greater in the thymus-grafted nudes prior to infection, whereas IgM levels were the same in both groups. Eleven days after infection, there were very large increases in IgG$_1$ and IgG$_2$ levels among thymus-grafted animals with only a comparatively modest increase in IgG$_2$ among the sham operated animals. IgM levels increased modestly in comparison in both groups. Antibody measured 11 days after infection showed only minimal differences between the two groups.

c. Spleen cells from "immune" BALB/c donors exhibited an in vitro proliferative response to 17XL P. berghei infected syngeneic RBCs and the crude antigen preparation. The response to P. berghei infected RBCs peaked on day 3 of in vitro cultivation, and that to the antigen preparation peaked on day 4. Normal cells responded only minimally to infected RBCs, peaking between 5 and 6 days, but did show a significant response to the antigen preparation between days 5 and 7 of culture, substantially later than that seen with immune cells. The response of both immune and normal spleen cells was T-dependent, as determined by sensitivity to pretreatment with anti-theta serum and complement. Similar proliferative responses were observed with lymph node cells from "immune" BALB/c mice, spleen cells from "immune" BLCF$_1$ mice, and spleen cells from thymus-grafted nudes after infection with 17XNL.

The in vitro proliferative response was demonstrated as early as 72 hours after infection with 17XNL in BALB/c mice. However, during the course of infection, there was a period during which both the in vitro P. berghei proliferative response and mitogen responses of spleen cells were depressed, correlating approximately with the period of active parasitemias. Both the antigen specific and mitogen responses were again observed after the infection was cleared (approximately 18-21 days). Removal of macrophages by passage over a Sephadex G10 column restored the mitogen responses to spleen cells during the period when they were depressed, but did not appear to increase the specific antigen responses to the same extent.

According to latest WHO figures, malaria, the world's most common disease, affects approximately 500 million people. Despite the existence of major differences between murine and primate malaria, the basic mechanisms by which mice cope with a blood induced infection may well be relevant to the human disease. Although large amounts of hyperimmune serum can confer protection in rodent malaria, this exceeds the amount of antibody demonstrated in the serum of an intact animal as it clears an initial nonlethal infection.

Likewise, although T-cell deprivation increases susceptibility to malaria, the exact subpopulations involved, and the mode of action, be it macrophage arming, helper function for antibody production, mediator production, etc., has not been elucidated. The development of an in vitro assay for T-cell sensitization provides a powerful tool for the analysis of the natural history of a T-cell response in malaria, and for the determination of the antigens to which T-cells respond. In the future, it may be possible to isolate those antigens to which T-cells become sensitized and use them as immunogens.

Immunosuppression is an important aspect of human and rodent malaria. The in vitro techniques provide means by which a detailed analysis of specific and nonspecific depression of immune responses can be observed and followed. It has previously been postulated that a B-cell mitogen accounts for the hyperglobulinemia of malaria. Our finding of a potent in vitro stimulator of normal cells that is T-dependent tends to refute this, although T-dependent B-cell stimulation may occur. The relationship of this presumptive T-cell mitogen to B-cell stimulation is under investigation.

The study of the natural history of the T-dependent in vitro proliferativ response during the course of infection with 17XNL is underway. It will be correlated with a delayed hypersensitivity response (footpad swelling), antibody measurement by IHA, parasitemeia, and T and B cell mitogen responses. We are also attempting physicochemical separation and purification of the antigens present in the crude freeze-thawed preparation. This will be analyze to determine which antigens react with antibody, and which trigger T cells in vitro. Cell transfer experiments are planned to determine which populations will reconstitute ATX-BM-ATS $BLCF_1$ mice. Finally, mice chronically injected with heterologous antiimmunoglobulin (anti-IgM) serum lack B-cells (as detecte by immunofluorescence) and are unable to mount demonstrable antibody responses. The course of a 17XNL infection will be studied in such mice.

Keyword Descriptors:

Thymus-derived (T) cells
Cell-mediated immunity
In vitro proliferative response
Malaria
Murine malaria
Mitogens, response in malaria
Nude (athymic) mice
Plasmodium berghei
Resistance to malaria
Immunoglobulin levels, in murine malaria

Publications: None

Project No. Z01 AI 00142-03 LMI
1. Microbial Immunity
2. Microbiology & Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Development of thymus-derived (T) suppressor and amplifier cell function

Previous Serial Number: 119A

Principal Investigator: Phillip J. Baker

Other Investigators: Herbert C. Morse III, Benjamin Prescott, Philip W. Stashak, and Diana F. Amsbaugh, NIAID

Cooperating Units: None

Man Years:

Total: 13/12
Professional: 7/12
Others: 6/12

Project Description:

In order to determine the time, after birth, at which suppressor and amplifier T cell activity is first demonstrable, BALB/c AnN mice, 2-8 weeks of age ± 3 days, were given a single injection of different amounts of Type III pneumococcal polysaccharide (SSS-III) to establish the dose of SSS-III required to elicit maximal numbers of antibody-producing plaque-forming cells (PFC); PFC making antibody specific for SSS-III were detected by an adaptation of the technique of localized hemolysis-in-gel. The degree of unresponsiveness induced in mice, previously given a marginally immunogenic dose of SSS-III (low-dose paralysis or tolerance) was used as an index of suppressor T cell activity for each age group considered; the magnitude of enhancement produced in mice given antilymphocyte serum (ALS) at the time of immunization with SSS-III, enabled one to assess the activity of amplifier T cells, in the absence of suppressor T cell function.

Dose-response studies for mice of different ages showed that the magnitude of the antibody (PFC) response to SSS-III increased with the dose of antigen used to immunize, until maximal values were attained in mice given an optimally immunogenic dose; this dose was found to be 0.05 μg, for mice 2 weeks of age, and 0.5 μg for all other age groups considered. The ability of mice to give a PFC response to an optimally immunogenic dose increased rapidly with age, reaching adult levels at 4 weeks of age; at 2 and 3 weeks of age, the magnitude of the PFC response produced was 7% and 40%, respectively, of that found in adult mice. Suppressor T cell activity, as measured by the degree of low-dose paralysis induced (90-95%), was evident in mice, 2-3 week of age; however, amplifier T cell activity was minimal at this age, and increased in a sigmoid fashion until maximal activity was achieved at 8-10 week of age.

Previous studies in this laboratory showed that suppressor T cells act primarily by limiting the extent to which bone marrow-derived precursors of antibody-forming cells (B cells) proliferate in response to antigen, and that suppressor T cells play an important role in the development of immunological paralysis (tolerance). Since suppressor T cells are present early in life ar can be activated at a time when amplifier activity is minimal and B cells are not capable of responding fully to some antigens, they may contribute to the ease with which tolerance is induced in neonates and act to suppress the development of antibody responses to self-antigens, i.e., auto-immune disease Additional info concerning the development of suppressor T cell activity woul be of great value in evaluating the risks (if any) involved in the administration of certain vaccines to children.

The dominance of suppressor T cell activity in young animals suggests that these animals might provide an ideal model for investigating the mechanism by which suppressor T cells are activated, thereby permitting one to assess more fully their role in the development of tolerance and the prevention of auto-immune disease.

Keyword Descriptors:

Thymus-derived suppressor cells
Suppressor T cells
Thymus-derived amplifier cells
Amplifier T cells
Type III pneumococcal polysaccharide (SSS-III)
Tolerance
Immunological paralysis
Immunological unresponsiveness
Low-dose paralysis
Low-dose tolerance
Ontogeny of the immune response
Antilymphocyte serum (ALS)
Antibody-producing cell
Plaque-forming cells (PFC)
Bone marrow-derived prevursors of antibody-forming cells (B cells)
Auto-immune disease

Honor and [illegible]

Publications:

Baker, P.J., Stashak, P.W., Amsbaugh, D.F., and Prescott, B.: Regulation of the antibody response to Type III pneumococcal polysaccharide. IV. Role of suppressor T cells in the development of low-dose paralysis. J. Immunol. 112: 2000, 1974.

Baker, P.J., Prescott, B., Stashak, P.W., and Amsbaugh, D.F.: Regulation of the antibody response to Type III pneumococcal polysaccharide by thymus-derived cells. In Sercarz, E.E., Williamson, A.R., and Fox, C.F. (Eds.): The Immune System: Genes, Receptors, and Signals. New York, Academic Press, Inc., 1974, pp. 415-

Baker, P.J., Burns, W.H., Prescott, B., Stashak, P.W., and Amsbaugh, D.F.: In Katz, D.H. and Benacerraf, B. (Eds.): Immunological Tolerance: Mechanisms and Potential Therapeutic Applications. New York, Academic Press, Inc., 1974, pp. 493-

Project No. Z01 AI 00143-06 L
1. Microbial Immunity
2. Microbiology & Immunol
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Genetic control of the antibody response to Type III pneumococcal polysaccharide (SSS-III)

Previous Serial Number: 119A

Principal Investigator: Phillip J. Baker

Other Investigators: Benjamin Prescott, Philip W. Stashak, and Diana F. Amsbaugh, NIAID

Cooperating Units: None

Man Years:

Total:	9/12
Professional:	3/12
Others:	6/12

Project Description:

Strains of inbred recombinant mice were evaluated with respect to (a) the magnitude of the antibody-producing plaque-forming cell (PFC) response produced after immunization with an optimally immunogenic dose of SSS-III, (b) the degree of unresponsiveness (tolerance), induced after pretreatment with a marginally immunogenic dose of SSS-III, which provides a measure of suppressor T cell activity, and (c) the degree of enhancement obtained upon immunization with SSS-III and treatment with antilymphocyte serum (ALS), which provides an index of amplifier T cell activity in the absence of suppressor T cells. Statistical comparisons were made to establish whether the results obtained could be associated with the allotype or the major histocompatibility (H-2) type of the strains of mice employed.

Previous studies in this laboratory have shown that, in addition to an X-linked gene that governs responsiveness to SSS-III in an almost "all-or-none" manner, several autosomal dominant genes, perhaps acting at different levels of the immune response, appear to influence the magnitude of the antibody response to SSS-III. The present work clearly illustrates that the ability of mice to give an antibody response to SSS-III, the activity of suppressor T cells, and the activity of amplifier T cells, are not associated with either allotype, or the major histocompatibility (H-2) complex of mice. Furthermore, responsiveness to SSS-III is not associated with the ability of mice to respond to other polysaccharide antigens, e.g., dextran, C polysaccharide, and bacterial lipopolysaccharides.

Cell-cooperative interactions have been found to be essential for the development of a normal antibody response to several well-studied antigens. Some investigators have reported that H-2 associated immune response (Ir) genes play an important role in mediating cell-cooperative interactions for antibody responses to synthetic (non-biological) antigens. However, such cell-cooperative interactions are not essential for a normal antibody response to SSS-III. The present work indicates that H-2 linked Ir genes - and their products - are distinct from those mediating suppressor and amplifier T cell functions. Although the immediate objectives of this project have been achieved, additional studies on the genetic control of the antibody response to SSS-III will be resumed as appropriate genetic models become available. The ultimate goal of this work will be to identify certain genetic traits or markers that either influence the development of a normal immune response, or are suggestive of certain immunological abnormalities.

Keyword Descriptors:

Inbred recombinant mice
Plaque-forming cells (PFC)
Suppressor T cells
Antilymphocyte serum (ALS)
Amplifier T cells
Low-dose paralysis
Low-dose tolerance
Tolerance
Histocompatibility (H-2) complex
Allotype
Genetic control
Immune response (Ir) genes

Honors and Awards: None

Publications:

Amsbaugh, D.F., Hansen, C.T., Prescott, B., Stashak, P.W., Asofsky, R., and Baker, P.J.: Genetic control of the antibody response to Type III pneumococcal polysaccharide. II. Relationship between IgM immunoglobulin levels and the ability to give an IgM antibody response. J. Exp. Med. 139: 1499, 1974.

Baker, P.J.: Homeostatic control of antibody responses: A model based on the recognition of cell-associated antibody by regulatory T cells. Transplant. Revs. (in press).

Project No. Z01 AI 00144-11 LM
1. Microbial Immunity
2. Microbiology & Immunolog
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of the antibody response to Type III pneumococcal polysaccharide

Previous Serial Number: 119A

Principal Investigator: Phillip J. Baker

Other Investigators: Jeffrey M. Jones, Benjamin Prescott, Philip W. Stashak, and Diana F. Amsbaugh, NIAID.

Cooperating Units: None

Man Years:

Total:	13/12
Professional:	7/12
Others:	6/12

Project Description:

A relatively simple procedure was developed for the preparation of tyramine Type III pneumococcal polysaccharide (tyramine-SSS-III), which then could be radio-labeled with ^{125}I to give a product of high specific activity (2,000 cpm/ng), without changing its immunological and physical characteristics. This material was used (a) in a radio-immunoassay to obtain more precise information concerning the amount of serum antibody produced at various intervals following immunization with different amounts of SSS-III, and (b) to examine the relationship between the distribution of SSS-III in the serum and tissues of immunized mice and the kinetics for the appearance of serum antibody and antibody-producing plaque-forming cells (PFC).

The ^{125}I-labeled-SSS-III so prepared was capable of detecting nanogram amounts of serum antibody and antigen in the tissues of immunized mice, and was especially useful in monitoring the excretion of SSS-III and determining whether degradation takes place in vivo. When mice were placed on a low residue diet, almost all of the injected ^{125}I-labeled-SSS-III could be recovered in the urine during the first 48 hrs post injection; some degradation of antigen occurred in vivo. This observation is consistent with the fact that large quantities of SSS-III can be detected in the urine of patients with lobar pneumonia. The administration of >100-fold molar excess of unlabeled SSS-III failed to replace significant amounts of ^{125}I-labeled-SSS-III taken up by cells of the reticulo-endothelial system; furthermore, the amount present in the circulation of mice given an optimally immunogenic dose of

SSS-III was too small to influence serum antibody levels as measured by the Farr test. These findings clearly show that the peripheral neutralization of antibody by excess recirculating antigen is not an important factor in the immune response to an optimally immunogenic dose of this antigen; studies on the rate of elimination of passively administered antibody in mice given different amounts of SSS-III affirmed this view.

The results of other studies in this laboratory suggest that thymus-derived amplifier cells (amplifier T cells) influence the rate of antibody synthesis and release by PFC. The radio-immunoassay used in the present work should enable one to obtain more precise information on the amount of antibody made following immunization with SSS-III; this information, in conjunction with data already available on the numbers of cells participating in the antibody response to SSS-III would permit more exact measurements on the effects of immunizing dose on the rate of antibody synthesis at the cellular level. Particular attention will be placed on the effects of tolerance-inducing doses of SSS-III on this process, and in obtaining information concerning the mechanism for central failure of the immune response that accompanies the development of a tolerant state.

Keyword Descriptors:

- Tyramine-SSS-III
- ^{125}I-labeled SSS-III
- Type III pneumococcal polysaccharide (SSS-III)
- Radio-immunoassay
- Farr test
- Treadmill neutralization of antibody
- Tolerance
- Central failure of immune response
- Immunological paralysis

Honors and Awards: None

Publications: None

Project No. Z01 AI 00145-08 LM
1. Microbial Immunity
2. Microbiology & Immunolog
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mode of action of thymus-derived (T) suppressor and amplifier cells

Previous Serial Number: 119A

Principal Investigator: Phillip J. Baker

Other Investigators: Jeffrey M. Jones, Benjamin Prescott, Philip W. Stashak, and Diana F. Amsbaugh, NIAID

Cooperating Units: None

Man Years:

Total:	13/12
Professional:	7/12
Others:	6/12

Project Description:

In order to define the mechanism by which suppressor T cells influence the magnitude of the antibody response to Type III pneumococcal polysaccharide (SSS-III), mice were immunized with an optimally immunogenic dose of antigen and assayed at 2-hr time intervals for antibody-producing plaque-forming cells (PFC) making antibody specific for SSS-III; the kinetic data obtained were compared to those of mice, depleted of suppressor T cells by treatment with antilymphocyte serum (ALS).

The results showed that the exponential increase in PFC proceeded in a stepwise fashion and involved multiple non-random recruitment from a precursor cell population. PFC first appeared 30 hrs after immunization; however, treatment with vinblastine (a mitotic inhibitor) revealed that the proliferation of precursor cells actually began 12-16 hrs prior to the appearance of such PFC. Maximal numbers of PFC, achieved at about 85 hrs after immunization, were maintained for nearly 15 hrs before declining. Increases in serum antibody concentration followed increases in PFC counts by 16-24 hrs during the first 60 hrs of the immune response, but maximal serum antibody concentrations were attained 40-48 hrs after maximal numbers of PFC were found. When mice were treated with ALS at the time of immunization with SSS-III, the stepwise exponential increase in PFC resembled that of non-ALS-treated mice during the first 100 hrs after immunization. Thereafter, additional stepwise increases in PFC occurred, resulting in a 20-fold increase in PFC counts.

These observations, in addition to those described in other studies (see last year's annual report) leave no doubt that suppressor T cells act primarily by limiting the extent to which antibody-forming cells proliferate following immunization; further information on the mechanism by which this occurs would provide insight concerning the basis for several lympho-proliferative disorders and for controlling undesirable immune reactions in vivo. Studies will be conducted to determine how suppressor T cells become activated during the course of an immune response, and how they function to limit the proliferative response of lymphoid cells.

Keyword Descriptors:

Suppressor T cells
Amplifier T cells
Proliferative response
Antilymphocyte serum ALS
Lympho-proliferative disorders

Honors and Awards: None

Publications: None

Project No. Z01 AI 00146-2 LMI
1. Microbial Immunity
2. Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Chemical and biochemical studies on biologically active fractions isolated from various plant and bacterial sources

Previous Serial Number: 70C

Principal Investigator: Benjamin Prescott

Other Investigators: George Caldes, LMI, NIAID
Robert M. Chanock*
Charles M. Helms*

Cooperating Units: *Laboratory of Infectious Diseases, NIAID, NIH

Man Years:

Total:	24/12
Professional:	12/12
Others:	12/12

Project Description:

I. *Lipid*.

Updating earlier research with plant glycolipids, we showed that there is a digalactosyl diglyceride present in plant material that cross reacts with antibodies to *Mycoplasma pneumoniae* in the complement-fixation and growth inhibition tests (Project 64D, 1974). The presence in plants of a higher homologue trigalactosyl diglyceride has also been indicated (Kenny and Newton, Annals N.Y. Acad. Sciences 225, 54-61 (1973). We were interested in isolating this compound from lipid extracts (chloroform-methanol 2:1 v/v) from carrots in order to reaggregate the purified compound with *Acholeplasma laidlawii* membrane protein for the production of a hybrid reaggregate which might be more effective than the digalactosyl diglyceride reaggregate in stimulating local defense mechanisms in the respiratory tract, prevent disease in man caused by *M. pneumoniae* and a possible source of a reproducible immunizing agent for use in a potential vaccine.

The trigalactosyl diglyceride was isolated from a chloroform-methanol (2:1 v/v) extract of carrots and purified by a combination of chromatographic methods previously employed for the isolation of digalactosyl diglyceride (Project 64D, 1973 and 1974). The compound exhibits high levels of activity in the complement fixation test with *M. pneumoniae* antiserum. The purified trigalactosyl diglyceride compound isolated from carrots has been chemically and physically identified by elemental analysis, hydrolysis and gas chromatographic analysis. The results of the analytical studies have indicated that the hapten contains glycerol, fatty acids and carbohydrates which are attached to glycerol through ester bonds, with galactose as the only carbohydrate component in a ratio of galactose : fatty acids : glycerol of 3:2:1. Evidence obtained in the studies suggests that the trigalactosyl diglyceride is a higher homologue of mono- and digalactosyl diglyceride and contains an additional D-galactopyranosyl group bound in the α ($1 \rightarrow 6$) position to the end galactose component of the digalactosyl diglyceride. The fundamental structural similarity in the carrot glycolipids with those of *M. pneumoniae* glycolipids is that they both contain galactose moieties which may be responsible for the serological cross reactivity, suggesting that the galactose may be the common antigenic determinant. Further studies of the properties and reactions of the trigalactosyl diglyceride may be of considerable interest in the possible role of this glycolipid hapten in pathological and immunological reactions.

II. Polysaccharides: Chemical and Biochemical Studies of Certain Pneumococcal Polysaccharides with Special Reference to their Biological and Immunological Properties.

A. The occurrence of polysaccharide antigens in the higher type pneumococci makes it probable that the compounds fulfill a structural role and may be of some immunological significance. To date, it has been shown that certain of the higher types (6, 7, 14, 18, 19, 23) pneumococcal polysaccharides are not very effective immunogens in animal and man. Recent studies in our laboratory in carbohydrate chemistry have provided us with an insight into the carbohydrate composition and structure present in certain of these higher types. All of the higher type organisms studied yielded polysaccharides in varying amounts when grown in trypticase soy broth. The isolation of the polysaccharides was carried out by a calcium phosphate precipitation procedure. Purification of the initial polysaccharide through repeated alcohol precipitations led to products free of protein. It was felt that perhaps development of new approaches such as chemical modification of the molecular structure of the polysaccharides might lead to analogs of the polysaccharides antigenically more active than the natural material. Accordingly, development of a variety of chemical methods are being carried out for incorporating functional groups onto the hydroxyl groups of the polysaccharides, such as introducing acetyl groups with acetic anhydride in the presence of pyridine, aryl carbamate substituents utilizing various isocyanates and adding certain proteins. Chemical, physical and biological properties of the new compounds will be studied to determine if these procedures may be effective means of increasing the immunogenicity of the polysaccharides.

B. Collaborative studies were carried out with Dr. Charles Helms of the Laboratory of Infectious Diseases on the chemical and biological properties of polysaccharides isolated from six temperature-sensitive mutants of Type I *Streptococcus pneumoniae*. Chemical and physicochemical studies (nitrogen content, carbohydrate composition, optical rotation, precipitin titer, antibody nitrogen) indicate that the synthesis of the polysaccharides in the ts mutants produces immunochemically reactive Type I capsular polysaccharide similar to that present in the parent organism. In addition, in *in vivo* studies in mice, it was possible to show that there appeared to be no demonstrable differences in immunogenicity between the various ts polysaccharides and the parent polysaccharide.

C. The method that was used in isolating and purifying the pneumococcus polysaccharides was adapted to the isolation of polysaccharides from Streptococcus mutans, Leuconostoc mesenteroides, Aspergillus niger and certai plants to be studied for their effect on cells of the immune system.

III. Chemotherapy of Potential Anticancer Agents from Natural Products. A systematic investigation of potential chemotherapeutic agents in 40 plants indigenous to Greece has revealed that the elaboration of anticancer agents is a characteristic property of several groups of plant species. The number of species studied represents a wide range of plants of unrelated chemical constitution. Aqueous extracts of the plants were prepared and purified by alcohol fractionation and DEAE-cellulose column chromatography. The products were tested for antitumor activity in two tumor systems (Walker 256 carcinosarcoma intramuscular) and lymphocytic leukemia P388 by screeners under contract to the Cancer Chemotherapy National Service Center. The testing procedures employed for the Walker 256 have been described in Protocol 1.502 Cancer Chemotheraphy Reports No. 25 (1962) and for P388 in Lymphocytic Leukemi Protocol Code 3PS (1968. Two of the extracts studied (Globularia alypium and Carthemus tinctorus) showed good inhibition against the lymphocytic leukemia P388 mouse tumor system; this activity was confirmed. Screening data for the Walker 256 showed no significant antitumor effects. Synthesis of organic compounds (thiosemicarbazones and hydrazones) with the purpose of obtaining no toxic compounds with high chemotherapeutic activity against tumors, malaria and viruses. Several compounds in the α-methylchalkone series showed confirme activity against Plasmodium berghei in experimentally infected animals and against Walker 256 carcinosarcoma in the rat tumor system.

IV. New Procedure. Because of the large number of analyses of carbohydrates on a wide range of natural products on paper chromatograms developed in our laboratory and the failure of the methods in use to detect the carbohydrate trehalose, a non-reducing disaccharide consisting of two glucose residues on the chromatograms, we developed a new sensitive procedure for detection of this carbohydrate on paper chromatograms. The method is as follows: A very active trehalase is known to be present in honeybees. Spraying the carbohydrate chromatograms with an aqueous extract of honeybees (Apis millifera) before treatment with the aniline salt reagent yielded sufficient color so that trehalose could be identified and estimated along with the other carbohydrates in a mixture. The trehalose is readily separated from the common monosaccharides and from sucrose, maltose and saffinose by ethyl acetate-pyridine-water (180:50:57.5) on Whatman #4 paper.

Keyword Descriptors:

Analytical analysis
Anticancer
Antigens
Chromatography
Immunological activity
Precipitins
Polysaccharides

Publications:

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LABORATORY OF PARASITIC DISEASES

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Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

SUMMARY - July 1, 1974 through June 30, 1975

INTRODUCTION

The turnover in professional personnel included incoming new Research Associates, Drs. Mason, Weintraub and Weller and two new Staff Fellows, Drs. Chen and Gottlieb. Dr. Kuberski, a former Associate, rejoined our Pacific Research Section as a Staff member. Also joining us as a Visiting Research Associate from the University of Edinburgh was Dr. Carter. Departing in 1975 were Senior Staff Fellow, Dr. Pertel, and Associates Marino, Raizman, and Shanley. Dr. Wyler will leave for a year of clinical training but is scheduled to return to LPD in 1976. Dr. Gubler, guest worker with Pacific Research Section will be going to Indonesia but will continue to collaborate with Dr. Rosen's group in Hawaii.

Except for annoying renovations elsewhere in Building 8, the new facilities for the Malaria Section in this structure have proved very adequate. The fact that the new insectary has functioned from the start without technical inadequacies or the usual mysterious problems is eloquent testimonial to the knowledge and foresight of all who participated in its design and planning. Thus far we have maintained a sense of both physical and intellectual togetherness in spite of LPD being in two buildings and we hope to keep it that way. Pacific Research Section also seems to thrive in its distant Hawaiian location perhaps becasue of it.

Several working assignments to foreign countries were carried out by LPD personnel. Dr. Wyler spent 15 weeks as a WHO consultant studying aspects of cell-mediated immunity in malaria at the British Medical Research Council's facility in The Gambia, West Africa. The out island of Mauke in the Cook Islands of the South Pacific was the site of a 3 man expedition for 4 months focused mainly on immunologic response in filariasis and staffed jointly by Drs. Ottesen and Heck of LCI (NIAID) and Weller of LPD. The cooperation of Dr. Chamberlayne of the Director's office in maintaining satellite-radio communications with the Cook Islands during the project was extremely helpful.

RESEARCH ACCOMPLISHMENTS

INVASION OF RED CELLS BY MALARIAL MEROZOITES The recent new focus upon factors governing invasion of red cells by malarial merozoites has yielded highly interesting information about this blood phase of the parasite. Direct observations and analysis of video-taped photographs of the invasion process has led to the hypothesis that after attachment of the merozoite to the red cell membrane and during invagination a temporary defect of the membrane permits entry of salt and water and results in cell swelling. After actual entry of the parasite the membrane lesion re-seals. Even red cell ghosts were found to be invaded by merozoites if high ATP concentrations were present. A movie of these events was made and is available from the National Library of Medicine. A surface coat that appears on merozoites after release from schizonts has antigenic characteristics which may be of functional importance since it has recently been shown by others that immunization with merozoite antigens produces a surprising degree of immunity. However, the most exciting discovery was the new insight into the immunochemical nature of the red cell receptor for *Plasmodium knowlesi*. This actually was first predicted by Miller from the association of frequency of Duffy negative blood group status in West Africans and U.S. blacks and their resistance to *P. vivax* malaria. Use of Duffy positive and negative red cells in an *in vitro* system with *P. knowlesi* then disclosed that the Duffy positive red cells were invaded whereas the Duffy negative cells were resistant. In addition, removal of Duffy blood group determinants by proteolytic enzymes or blocking the receptors with anti-Duffy serum markedly reduced invasion. These findings now open the way to a search for similar type receptors for other species of malaria, and to possible exploitation of this knowledge for other approaches to rendering red cells resistant to merozoite invasion as a method of treatment or prevention of malaria (Miller, Mason, Dvorak).

VARIOUS ASPECTS OF MALARIAL IMMUNOLOGY UNDER STUDY

Cases of falciparum malaria in an endemic region (The Gambia) were found to have significantly reduced circulating T cells during acute infection (Wyler). The finding of a chemotactic factor for monocytes in splenic extracts of mice and monkeys with malaria may help explain why splenomegaly is so prominent in malaria (Wyler and Gallin of LCI). A review of old data on the influence of splenectomy upon the course of established *P. inui* infections in monkeys has revealed the interesting observation that splenectomized animals cure themselves in contrast to intact animals that have chronic infection for many years (Wyler, Miller and Schmidt). A membrane-feeding system has been developed for studying the influence of various materials upon infectivity of gametocytes for mosquitoes. With this system serum from certain monkeys chronically infected with *P. cynomolgi* is inhibitory to infection in mosquitoes, suggesting some type of gametocyte-specific immunity (Gwadz). Techniques are being improved for cleaning up sporozoite preparations from mosquitoes and sporozoite induced immunity in both rodent and simian models is under study for the role of cell-mediated aspects of immunity (Chen and Gwadz). It is hoped that methodology of the schizont agglutination test used for studying antigenic variation in *P. knowlesi* malaria can be used to identify antigens on the surface of infected red cells and merozoites to indicate stages of the parasite responsible for malarial immunity (Powers).

GENETIC FACTORS INVOLVING VERTEBRATE AND ARTHROPOD HOST IN MALARIA

Rodent malaria in different genetic stocks of mice is being studied to determine the influence of the vertebrate host on various characteristics of the malaria parasite.

(Carter)

Now that a more predictable method for infecting mosquitoes has been developed via membrane feeding (see above) it will be possible to take up again the work on genetic factors regulating susceptibility and resistance of anopheline vectors to malaria (Gwadz).

CHEMOTHERAPY OF MALARIA

Studies with radio-labeled clindamycin showed no difference in uptake of the drug by resistant or sensitive parasites as is the case in resistance to chloroquine. A new antimalarial drug, mefloquine (a 4-quinolinemethanol), which has excellent suppressive activity against drug-resistant P. falciparum in man, is being studied in rodent malarias that are resistant to various drugs (Jacobs).

WORK ON HELMINTHS AND HELMINTHIC DISEASES

Schistosomiasis: Work of the past few years on genetic factors that influence whether a snail is susceptible to the parasite has led to similar questions being asked about genetic control over infective capacity of the schistosome for its molluscan host. Finding such answers requires uniform genetic stocks of snails so that this variable is held constant. Evidence was obtained that a St. Lucian strain of S. mansoni was a mixture of at least two varieties of schistosomes in terms of their infectivity for snails. What were once considered straight-forward experiments of schistosome-snail intereactions now turn out to be quite complicated and subject to various interpretations. The early host tissue response to miracidial penetration in refractory snails may be a useful clue as to the mechanism by which insusceptibility is expressed (Richards). Reassessment of the papillary bladder lesions previously observed in capuchin monkeys infected with S. hematobium is in order. Some of these lesions have regressed and autologous transplants of tumor did not grow, suggesting that some of the papillary tumors are not neoplastic. However, sessile bladder growths seen in other types of monkeys with schistosome infections show histologic features more compatible with malignant tumors (Cheever).

Filariasis: Antigens from several stages and species of filarial parasites have been prepared and tested for ability to transform lymphcoytes from the Mongolian jird, a good host for experimental infections with Brugia pahangi and B. malayi. This aspect of cell-mediated immunity will be followed in the jird. The same antigens were used in the human filariasis study carried out in the Cook Islands and reported elsewhere (Z01 AI 00047-06 LCI). Several inbred strains of rats are under test for susceptibility to infection with infective larvae of B. pahangi (Weller, Ottesen, Gwadz).

Free-living nematodes as models for genetic and biochemical studies: The structure and function of the nervous system in Caenorhabditis elegans, a free living nematode, was studied since it might reflect behavioral changes resulting from induced mutations. Neuronal cells producing serotonin and acetylcholinesterase were identified by histochemical and ultrastructural methods (Pertel, Mattern of LVD, Luban).

PATHOGENESIS AND IMMUNITY OF PROTOZOAL INFECTIONS

Toxoplasma and related coccidial organisms: Attempts to inhibit oocyst production without interfering with development of immunity to Toxoplasma gondii infection have been partially successful. When cats were infected and treated with a new anti-coccidial drug, lasolacid, antibody developed but oocysts, which are an infective form for man, were not excreted. Later challenge of the cats with a new infection likewise failed to produce oocysts. These observations suggest that it whould be possible to immunize against the intestinal phase of oocyst production in coccidial infections (Sheffield and Melton). Considerable uncertainty and confusion exists about the biologic and taxonomic status as well as medical importance of the coccidial group of organisms to which toxoplasma belongs, including Sarcocystis and the various isosporoses. Since these organisms commonly infect a variety of domestic animals, they may constitute a more important zoonotic potential than hitherto appreciated. For example, the organism first thought to be a Sarcocyst and now considered a new species of coccidia, Hammondi hammondi, (See Project Report Z01 AI 00102-01 LPD) has oocysts which are indistinguishable from T. gondii. This organism stimulates only a low level of antibody to toxoplasma, but has quite different biologic characteristics (Wallace, Sheffield and Neva). Another coccidian-type organism excreted and transmitted via cat feces has been shown to be a member of the genus Besnoitia (Wallace).

Further experience was gained with techniques for demonstrating circulating toxoplasma antigen in experimentally infected animals. The antigen can be purified and concentrated by aid of affinity columns of sepharose linked with antibody. Clinical specimens obtained in this manner and tested by counter immunoelectrophoresis have given suggestive results but will probably require a more sensitive assay procedure (Raisman and Neva).

Amebae and intestinal protozoa: Continued studies of the ameba viruses has disclosed a third variety of virus particle, and an increasingly complex virus-host cell relationship with some strains being more lytic than others and/or differences in host cell susceptibility to lytic infection (Mattern, Keister and Diamond). Virulence of cloned amebal strains, as assessed by ability to induce abscesses in the hamster liver by direct inoculation, could not be correlated with titer of associated virus. However, separate clones of a given strain of ameba showed significant differences in virulence by the hamster liver test (Marino and Diamond). The tissue response to axenically cultivated amebae, with and without microbial associates is being studies in the guinea pig and jird (Takeuchi of AFIP and Phillips). The possibility that antibody responses in specific immunoglobulin classes (IgA, IgM, etc.) might be helpful in serodiagnosis of different types of amebic infections is being examined (Lunde). Blastocystis homis, an organism generally considered a non-pathogen in man was found to produce invasive lesions and diarrhea in germfree guiena pigs (Phillips and Zierdt of Clinical Center).

Trypanosomiasis: While previous studies of interaction of T. cruzi with host cells in vitro had focused upon penetration and later events within the cell, attention more recently was directed to an earlier phase of possible attraction of parasites to host cells. With the aid of electrocptical instrumentation to quantify results it was found that various cells have 2 to 10-fold differences in ability to attract trypomastigotes of T. cruzi. This might be one explanation for the initial negative binominal distribution of parasites among a population of host cells (Dvorak). The attraction of certain cells or tissues for this parasite may also correlate with the pattern of plasma isoenzyme changes observed in mice infected with T. cruzi. In general, the elevated plasma LDH isoenzymes reflect pathologic changes sustained by tissues such as liver, heart, and muscle. Use of more tissue-specific isoenzyme assays should permit this type of analysis to characterize tissue tropism of various strains of *T. cruzi* (Mercado).

Requirements of intra-cellular replication of *T. cruzi* have been examined under conditions of an anti-parasitic drug and with restricted nutrients. The maximum concentration of Lampit, a nitrofurfurylidene (Bayer 2502) tolerated by *T. cruzi* in cells was determined to be about 10^{-5}M, with lengthened reproduction time but continued viability at 10^{-6}M. A strain of parasite with acquired and stable resistance to the drug was produced. A strain of *T. cruzi* was adapted to growth in vertebrate cells using a serum-free cell culture medium (Dvorak).

Leishmaniasis: A reproducible model of leishmanial infection (*L. tropica*) in a genetic inbred strain of mice has been established which permits the study of many different aspects of this infection. The dose-response characteristics have been quantified, the infection is progressive with visceralization to liver and spleen, and amastigotes can be harvested from lesions. Parasite-macrophage interactions *in vitro* are under study and a more detailed genetic analysis of mouse susceptibility is being examined (Weintraub and Neva).

PARASITE BIOCHEMISTRY AND MECHANISMS OF ENERGY METABOLISM

In amebae: The continued availability of axenically cultivated *E. histolytica* from Dr. Diamond's group has permitted further productive collaboration on the respiration and metabolism of this organism. Three separate lines of evidence have clearly shown that iron-sulfur proteins are functional in electron transfer of the axenized organism: (1) respiration was inhibited by metal-chelating agents, (2) analytical data on content of non-heme iron and acid-labile sulfide and (3) the characteristic signal for non-heme iron obtained in intact trophozoites by para-magnetic resonance spectroscopy (Weinbach, Diamond and Kon of NIAMDD). Studies on the oxidation of L-serine by *E. histolytica* has shown that this amino acid is metabolized in a complex fashion, including participation of two types of pyruvate oxidases. One of these is coenzyme A-dependent and shows unique catalytic and molecular properties (Takeuchi and Weinbach).

Trypanosomes: The techniques to be employed for studying protein synthesis and polysaccharide metabolism in trypanosomes are first being developed using the insect trypanosome, *Crithidia fasciculata*. The polysaccharide component of this organism has been isolated and shown to be composed primarily of mannose. Experiments on labeling of the polysaccharide with ^{14}C sugars have shown that the material is probably a structural component and not an energy reserve (Gottlieb).

Tapeworms: With the new Dual-wavelength Spectrophotometer it has been possible to examine characteristics of the cytochromes in mitochondria of the rat tapeworm, *Hymenolepis diminuta*. The cestode mitochondria were found to contain an unusual cytochrome *b*, with no evidence for the presence of cytochromes *a* and *c* which are found in other animal cell mitochondria (Harlow).

Energy metabolism of blood platelets: A highly active diaphorase was found in blood platelets which catalyzes the oxidation of reduced pyridine nucleotides, NADH and NADPH. The enzymes may be associated with the oxygen burst which occurs in platelets after stimulation with thrombin. Even "outdated" platelets from the blood bank are suitable for some of the studies with this enzyme (Weinbach and Harlow).

DENGUE AND OTHER ARBOVIRUS INFECTIONS

Fatal dengue virus infections in the Pacific area: The introduction of dengue type 1 virus to Pacific islands (Nauru, Ponape and Fiji) after a 30 year absence afforded an opportunity to study the behavior and manifestations of this infection under relatively simple and known epidemiologic conditions. Observations are also available on a flare-up of type 2 dengue that occurred in Tonga and Tahiti. It was shown that fatal disease (hemorrhagic and shock syndrome) can result in individuals who have not experienced previous dengue infections.

In collaboration with the SEATO laboratory in Thailand it was demonstrated that 2 to 3 times as many virus isolations were obtained from dengue hemorrhagic fever patients by the mosquito inoculation technique than by conventional cell culture methods (Rosen, Gubler, Kuberski and various Pacific collaborators).

Vector competence for dengue viruses: Further studies of oral susceptibility to dengue virus among geographic strains of A. albopictus disclosed a similar pattern with all 4 dengue serotypes. A search for genetic differences in susceptibility of mosquito strains showed that significant changes resulted from selective inbreeding, but no clear cut mode of inheritance could be determined. A simple, direct feeding technique was developed to test the ability of mosquitoes to transmit dengue viruses without feeding on a vertebrate host (Gubler and Rosen).

Arboviruses other than dengue: Although different geographic strains of Aedes albopictus from the Pacific region showed variable susceptibility to infection with Chikungunya virus, resistance could not be readily manipulated by genetic selection. While serum surveys of Southeast Asia and Pacific islands show human Chikungunya virus infections confined to the mainland, Indonesia and the Phillipines, antibodies to another group A virus called Ross River were found in New Guinea, the Bismarck and Solomon islands. Evidence for human infection with 4 Phlebotomus fever group viruses was found in Greece, Iran, Egypt, Sudan and Nigeria using a plaque reduction neutralization test. This test for sandfly fever antibodies appears to be type-specific and the most sensitive for detection of antibodies to this group of viruses (Tesh).

STUDIES ON OTHER INFECTIOUS DISEASES OF THE PACIFIC AREA

Natural history of influenza in swine: Because of accumulated evidence suggesting the participation of animals in the epidemiology of human influenza infections and yet unresolved controversy over the mechanism(s) by which swine influenza is maintained and introduced into herds, studies on the natural history of swine influenza have been initiated at Pacific Research Section (PRS) by Dr. Wallace. This seems appropriate since PRS personnel have experience with both viral and parasitic infections affecting both animals and man and because isolated herds of swine are desirable for such studies. The findings may also have considerable relevance to human influenza. Antibodies to classical swine influenza A virus or human Hong Kong type A were found to be present in 45% of animals from 65 herds on the island of Oahu. Five herds were selected for further study and search is being made for presence of swine lungworms, which was postulated by Shope to serve as an extra-mammalian reservoir of virus (Wallace and Shanley).

Veneral diseases in Hawaii: A study on the etiology of non-gonococcal urethritis has been initiated in collaboration with a Navy group in Hawaii. The study is designed to search for bacteria, viruses and chlamydiae, mycoplasmas, as well as trichomonas (Kuberski and Shaw of the Navy).

Serologic studies on Angiostrongylus: A study has been initiated to utilize counter-immunoelectrophoresis for diagnostic purposes to detect antigens of *Antiostrongylus*, the causative agent of eosinophilic meningitis, in spinal fluid. In preliminary tests this method detected antigen in the serum of infected rats but none was found in the spinal fluid of rats or human cases (Kuberski).

HONORS AND AWARDS

The more siginificant honors received by LPD staff members in the past year included the following:

Dr. Sheffield served as Editor of the Proceedings of the Helminthological Society of Washington and was Chairman of the Section on Life Cycles of Coccidia, Toxoplasmidae and Sarcocystidae of the Third International Congress of Parasitology in Munich.

Dr. Dvorak was an invited participant and presented a paper at the PAHO International Symposium on Chagas' disease in Horizonte, Brazil.

Dr. Diamond was awarded the Superior Service Medal by DHEW; was elected Vice-Chairman of the Board of Trustees of the American Type Culture Collection, was appointed to the Executive Council of the Organizing Committee of the International Conference on Amebiasis, Mexico City; (General Parasitology) and was a member of the Editorial Board of Cryobiology.

Dr. Neva was an invited participant to a special meeting on schistosomiasis called by the Board on Science and Technology for International Development of the National Academy of Sciences; was reappointed as Visiting Lecturer, Harvard University School of Public Health; was a member of the Ad Hoc Study Group on Parasitic Diseases, Walter Reed Army Institute of Research and was a member of the Editorial Board of the Journal of Infectious Diseases.

Dr. Weinbach was invited to serve as a panelist on the Proposed Contract between the Association for the Advancement of Aging Research and the Secretary of DHEW.

Dr. Cheever continues to serve on the Tropical Medicine and Parasitology Study Section of NIH.

Dr. Miller is a member of the Ad Hoc Study Group on Parasitic Diseases, WRAIR; was a member of the Steering Committee and a participant in a workshop on vaccines for malaria for the National Research Council and was a participant in a workshop on the immunology of malaria of the World Health Organization in Geneva, Switzerland.

Mr. Phillips was reelected to the Board of Directors, Association for Gnotobiotics.

Dr. Richards and Dr. Tesh presented invited papers at the Conference on Pathobiology of Invertebrate Vectors of Disease sponsored by the New York Academy of Sciences.

Dr. Gubler was a short term consultant on filariasis in Jakarta for the World Health Organization.

Dr. Wallace was appointed as Associated Clinical Professor at the University of Hawaii School of Medicine and gave invited papers at the International Congress of Parastiology in Munich.

Dr. Rosen was elected President of the American Society of Tropical Medicine and Hygiene; was appointed representative of this Society and of the U.S.A. on the Executive Group of the Internation Interim Committee of the Internation Congress of Tropical Medicine and Malaria; was reappointed as Consultant to the Commander, U.S. Army Medical Research and Development Command and as a member of the Ad Hoc Study Group on Viral and Rickettsial Disease, WRAIR; was a member of the Editorial Board of Intervirology; was a member of the Executive Committee of the American Committee on Arbo-viruses; and was appointed as Clinical Professor at the University of Hawaii School of Medicine.

Serial No. Z01 AI 00091-13 LPD
1. Parasitic Diseases
2. Host-Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The biology and control of the snail intermediate hosts of schistosomes.

Previous Serial Number: NIAID 121 C

Principal Investigator: Charles S. Richards

Other Investigators: Dr. Margaret Kasschau, Guest Worker

Cooperating Units: Dr. Edward H. Michelson, Department of Tropical Public Health, School of Public Health, Harvard University.

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	30/12			30/12
Professional	12/12			12/12
Other	18/12			18/12

Project Description:

The objective is to study molluscan and trematode genetics with particular attention to applications to control measures. To study prospective agents for biological control of intermediate hosts of the schistosomes. To study the biology of the snails with the aim of improving the efficiency of control measures. To study the contributions and complications of molluscan diseases and molluscan genetics to bio-medical research. To study the causes of tumors in mollusks. To study host-parasite interaction and molluscan cellular defence mechanisms.

Individual snails (Biomphalaria glabrata and Biomphalaria straminea) are exposed to various strains of Schistosoma mansoni, and selection and crossing experiments conducted to investigate further the genetic aspects of molluscan susceptibility to infection. Results of penetration of individual miracidia of S. mansoni in different snail types are observed and selection and crossing experiments conducted to investigate the genetic aspects of schistosome infectivity.

Genetic studies on infectivity in S. mansoni have resulted in selection from the parent St. Lucian strain of two sub-strains differing in infectivity. Exposures of established genetic stocks of B. glabrata to these sub-strains has revealed differences in susceptibility suggesting involvement of additional genetic susceptibility factors in the snails. Selection and crossing experiments with established refractory and susceptible genetic stocks of B. straminea have resulted in a third genetic type, juvenile susceptible but adult refractory.

Sections have revealed that in refractory B. glabrata the host tissue encapsulating response is initiated as early as 2 hours after miracidial penetration.

Four different types of proliferative amebocytic accumulations have been observed in four different clonal stocks of B. glabrata. Experimental studies suggest the involvement of genetic factors. In the course of these studies the anterior pericardial wall was identified as probably the major hemopoietic area in B. glabrata. The amebocytic accumulations provide a simple source for mono-layer preparations which survive for several weeks in tissue culture media. Although subcultures have not yet been obtained, the preparations should serve for studies on interaction between host tissue and intracellular parasites such as microsporida, amebae, bacteria, etc.

Establishing genetic stocks of B. glabrata, particularly different types of susceptibility to S. mansoni, provides material for bio-chemical, biological and cytogenetic intraspecific analyses. Such stocks have been provided on request to several other investigators.

Need for evaluation of the role of genetics in snail control continues. Refractory B. glabrata may prove useful in biological control of schistosomiasis by dilution or replacement of populations of susceptible snails in endemic areas. Progress in understanding causes of susceptibility or insusceptibility of B. glabrata to infection with S. mansoni accelerates the prospect of safe field testing of refractory snail stocks for control.

Establishing markers for some of the various linkage groups in B. glabrata increases our capacity to analyze invisible and complex genetic characters such as host-parasite relations.

Information (including histopathology) on a variety of tumors observed in B. glabrata, many of which are genetic, should help in analysis of tumors of mollusks collected in the field. Studies on the proliferative amebocytic accumulations in B. glabrata have revealed information on hemopoiesis about which little is known, provided a possible source for molluscan tissue culture, and may help in understanding the dynamics of the early interaction between molluscan host tissues and invading miracidia of _S. mansoni_.

Microsporida, amebae, and bacteria cause extensive damage in some snail hosts. These and other snail diseases need to be examined for potential human infection, and prospective use as agents in biological control of snails. It is essential to be aware of the occurrence of these infections and their relations with the snails and other parasites to plan, conduct, and interpret experimental research on snails with validity.

Results increasingly implicate genetics in host-parasite relations involving various parasites.

Genetic studies on _B. glabrata_ and _B. straminea_ will be continued to: a) seek additional genetic markers; b) expand our knowledge of linkage groups; c) characterize and establish stocks of the various susceptibility combinations of _B. glabrata_ and _B. straminea_ for _S. mansoni_ infections; d) test additional stocks of _B. glabrata_ and strains of _S. mansoni_ as available and e) continue studies of incompatibility between certain stocks of _B. glabrata_. Genetic studies on _S. mansoni_ will be continued to establish different genetic infectivity strains and elucidate the genetics of infectivity.

Plans have been initiated for research studies in collaboration with Dr. Frederico Barbosa, Professor of Epidemiology at the University of Brasilia. Laboratory and field trials will be carried on in Brazil to assess the potential genetic control of transmission of _S. mansoni_ by the use of genetic stocks of _B. glabrata_ and _B. straminea_ refractory to infection.

Studies will be continued on molluscan defence mechanisms and interaction between host snails and trematode parasites.

Studies on protozoan and bacterial infections in mollusks will be continued: including relations to the molluscan hosts, effects on other parasites, potential for human infection, and as a variable factor in experimental research.

Molluscan abnormalitites will be studied by fresh dissections and culturing, serial sections, genetic selection and crossing, and environmental manipulation to determine causal factors. Studies on the histopathology of molluscan tumors will be continued in collaboration with Dr. Michelson.

Key Words:

Schistosoma mansoni, _Biomphalaria glabrata_, biological control, genetic control, molluscan vectors of disease, molluscan host susceptibility,

trematode infectivity, genetics of mollusks, molluscan cellular defence mechanisms.

Publications:

Richards, C.S. Genetics of pigmentation in Biomphalaria straminea. Am. J. Trop. Med. Hyg. 24: 154, 1975.

Richards, C.S. Antler tentacles of Biomphalaria glabrata: Genetic studies. J. Invert. Path. 24: 49, 1974.

Richards, C.S. Shell mats associated with microspirida in Biomphalaria glabrata: Genetic studies. J. Invert. Path. 24: 337, 1974.

Richards, C.S. Everted preputium and swollen tentacles in Biomphalaria glabrata: Genetic studies, J. Invert. Path. 24: 159, 1974.

Richards, C.S. Genetic factors. In Marine Bioassays Workshop Proceedings. Marine Technology Society, pp. 123, 1974.

Richards, C.S. and Merritt, J.W. Variation in size of Biomphalaria glabrata at maturity. The Veliger. 17: 393, 1975.

Richards, C.S. Genetic factors in susceptibility of Biomphalaria glabrata for different strains of Schistosoma mansoni. Parasitology. 70: 231, 1975.

Richards, C.S. Genetic studies on variation in infectivity of Schistosoma mansoni for Biomphalaria glabrata. J. Parasit. 61: 233, 1975.

Richards, C.S. Genetic studies of pathological conditions and susceptibility to infection in Biomphalaria glabrata. Annals N.Y. Acad. Sci. (Accepted)

Michelson, E.H., and Richards, C.S. Neoplasms and tumor-like growths in the aquatic pulmonate snail Biomphalaria glabrata. Annals N.Y. Acad. Sci. (Accepted).

Serial No. Z01 AI 00092-09 LPD
1. Parasitic Diseases
2. Host-Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The pathogenesis of schistosome infections in mammalian hosts

Previous Serial Number: NIAID 121 H

Principal Investigator: Allen W. Cheever

Other Investigators: None

Cooperating Units: Southwest Foundation for Research and Education (Dr. Kuntz); Department of Pathology, Hospital Prof. Edgard Santos, Salvador, Brazil (Dr. Andrade); Department of Pathology, Cairo U. School of Med. (Drs. Kamel and Elwi); Department of Zoology, University of Georgia (Dr. Damian); Division of Geographic Medicine, Case Western Reserve (Drs. Warren and Mahmoud).

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	26/12			26/12
Professional	10/12			10/12
Other	16/12			16/12

Project Description:

The nature and pathogenesis of the lesions produced in mammalian hosts by schistosomes of medical importance are studied in man and experimental animals.

Papillary epithelial growths in the urinary bladder of capuchin monkeys infected with S. haematobium have regressed in several animals, and autologous transplants of tumor did not grow. Although designated as early cancers on morphological grounds, the available evidence suggests that most of these lesions are not neoplastic. On the other hand, sessile bladder tumors differing from the above papillary lesions have been seen in 1 of 6 cynomolgus monkeys infected with S. intercalatum. Epithelial atypia is greater in these lesions, and they appear to be infiltrating bladder submucosa, although no metastases or invasion of bladder muscle are present, so that the biological behavior of these lesions is not established.

Key Words:

Schistosomiasis, pathology, obstructive uropathy, bladder cancer, liver.

Publications:

Cheever, A.W. and Duvall, R.H. Single and repeated infection of grivet monkeys with Schistosoma mansoni: parasitological and pathological observations over a 31-month period. Am. J. Trop. Med. Hyg. 23: 884, 1974.

Sadun, E.H., von Lishtenberg, F., Erickson, D.G., Cheever, A.W., Bueding, E.E. and Anderson, J.S. Effects of chemotherapy on the evolution of schistosomiasis japonica in chimpanzees. Am. J. Trop. Med. Hyg. 23: 639, 1974.

Soliman, L.A.M., Cheever, A.W., Kuntz, R.E. and Myers, B.J. Lesions of bladder muscle in baboons and monkeys infected with Schistosoma haematobium. Tropenmed. Parasit. 25: 327, 1974.

Cheever, A.W., Torky, A.H. and Shirbiney, M. The relation of worm burden to passage of Schistosona haematobium eggs in the urine of infected patients. Am. J. Trop. Med. Hug. 24: 284, 1975.

Kuntz, R.E., Cheever, A.W., Myers, B.J., Young, S.W. and Moore, J.A. Calcification of the bladder and papillary tumors of the bladder and ureters in gibbons (Hylobates lar) infected with Schistosoma haematobium (Iran). Trans. Roy. Soc. Trop. Med. Hyg. In press.

Cheever, A.W., Young, S.W. and Shehata, A. Calcifications of Schistosoma haematobium eggs: relation of radiologically demonstrable calcification to eggs in tissues and passage of eggs in urine. Trans. Roy. Soc. Trop. Med. Hyg (In press).

Mahmoud, A.A.F., Cheever, A.W. and Warren, K.S. Streptozotocin-induced diabetes mellitus and the host-parasite relation in murine schistosomiasis mansoni. J. Inf. Dis. In press.

Sadun, E.H., Reid, W.A., Cheever, A.W., Duvall, R.H., Swan, K.G., Kent, K.M., Bruce, J.I., and von Lichtenberg, F. Effects of portacaval shunting on *Schistosoma japonicum* infection in chimpanzees: dissociation of pipesten fibrosis and glomerulopathy. *Am. J. Med. Hyg.* In press.

Nash, T.E. Localization of the circulating antigen within the gut of *Schistosoma mansoni*. *Am. J. Trop. Med. Hyg*. *23*: 1085, 1974.

Oya, H., and Weinstein, P.P. Demonstration of cobamide coenzyme in *Ascaris suum*. *50B*: 435, 1975.

Serial No. Z01 AI 00093-01 LPD
1. Parasitic Diseases
2. Host-Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Immunologic studies of filariasis and other nematodes

Previous Serial Number: None

Principal Investigator: Peter F. Weller

Other Investigators: Eric A. Ottesen and Robert W. Gwadz

Cooperating Units: Laboratory of Clinical Investigation, NIAID

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	4/12			4/12
Professional	4/12			4/12
Other				

Project Description:

Antigens from several stages and species of filariae were prepared for use in laboratory and field clinical studies. *Brugia malayi* and *B. pahangi* adult and microfilarial stages were harvested from jirds infected intraperitoneally. *Dirofilaria immitis* adult stages were obtained from infected dogs, and *D. immitis* microfilaria were collected by filtration of dog blood through plastic micropore filters. Saline extracts of homogeneates of both stages of all three species were utilized as antigens in field studies of *Wuchereria bancrofti*.

In vitro lymphocyte transformation using lymphoid tissue from the Mongolian jird was investigated as a preliminary to future studies of cell-mediated immunity in filarial infections of the jird. Standard lymphocyte transformation techniques were employed to study the response of jird splenic and lymph node lymphocytes to mitogen and nonfilarial antigen, and optimal conditions for such were established.

In an attempt to establish filarial infections in other laboratory animals suited for immunologic studies adult rats of two ages of the Brown Norway, Lewis and Fisher inbred strains were infected subcutaneously with the L3 stage of _B. pahangi_. Subsequent bleedings revealed only a few rats to have developed patent circulating microfilaremias. Continued bleedings are anticipated.

Key Words:

Filaria, nematode immunity, filarial antigens, lymphocyte transformation, _Dirofilaria immitis_, _Brugia_ species.

Publications:

Weinstein, P.P., and Highman, B. Infection of the jird, _Meriones unguiculatus_, with the filarial worm, _Dipetalonema viteae_: Central nervous system invasion and pathology. _J. Parasit_. _60_: 138, 1974.

Pacheco, G. Relation between the number of circulating microfilariae and the total population of microfilariae in a host. _J. Parasit_. _60_: 814, 1974.

Pacheco, G. Dirofilaria immitis: A review of present knowledge. In Harmison, L.T. (Ed.) _Research Animals in Medicine_, DHEW, 1973, pp 977-980.

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Serial No. Z01 AI 00094-16 LPD
1. Parasitic Diseases
2. Parasite Growth and Diff. Sect.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on _Entamoeba histolytica_ and other parasitic protozoa.

Previous Serial Number: NIAID 122 G

Principal Investigator: Louis S. Diamond

Other Investigators: David B. Keister, Joseph T. Marino, Carl F.T. Mattern, and Bruce P. Phillips

Cooperating Units: Laboratory of Viral Diseases, NIAID

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	57/12			57/12
Professional	26/12			26/12
Other	31/12			31/12

Project Description:

Studies characterizing the indigenous viruses of _E. histolytica_ by means of cross-infection experiments using cloned and uncloned donor and recipient amebal strain continues. Virus from each of 4 HM-1:IMSS strain clones readily infected uncloned HK-9 amebae, while virus from only 3 of 4 of these clones induced a lytic effect in all HK-9 clones tested. These observations provide evidence for the existence of individual differences in susceptibility to infection in amebal populations (Keister-Diamond).

Indigenous virus from cloned strain H-458:CDC amebae was found to have a broad amebal host range. Electron-microscope studies showed the virus induced in the susceptible amebal strains to be morphologically, homogenous polyhedral particles. However, when the virus induced in each susceptible strain was passaged several times through that strain and then retested for host range, the range of one of the induced viruses was found to be different from the parent stock suggesting the presence of more than one polyhedral virus in the parent (Keister, Mattern, Diamond).

Exposure of uncloned HK-9 amebae to lysates of uncloned HM-1:IMSS has induced two morphological viral types (polyhedral and filamentous) in the former. In similar studies performed with cloned cultures of the same species not only were these two forms observed, but in one combination of donor and recipient a third particle was seen. This viral-like particle superficially resembling a bacteriophage has also been observed in lytic debris resulting from the exposure of strain H-458:CDC to HM-1:IMSS (Keister, Diamond, Mattern).

The virulence of axenically cultivated clones of *E. histolytica*, representing 3 amebal strains, was evaluated by direct inoculation of the amebae into hamster liver. One HB-301:NIH clone was found to be as virulent as the parental uncloned stock, whereas, three other clones were significantly less virulent. The uncloned stock of strain HK-9 and 4 of its clones were relatively avirulent compared to the highly virulent parent stock. These results indicate that an amebal population is comprised of individuals differing in their invasive capacity (Marino, Diamond).

Of the four HB-301:NIH clones tested the most virulent also had a significantly higher titer of indigenous virus than the others. Successful efforts to increase viral titer of two low-virulence clones by exposure to BUDR or by superinfection with virus from a high titering clone did not result in increased virulence. Similarly, a spontaneous increase in virus production by one clone did not change its virulence. Thus, no correlation was found between the viral titers of the amebae and virulence (Marino, Diamond).

Key Words:

Entamoeba histolytica, axenic cultivation, cloned strains, amebal viruses, viral cross-infections, bromodeoxyuridine, biochemical virus induction, amebal virulence assay, hamsters, electron microscopy.

Publications:

Diamond, L.S., Phillips, B.P. and Bartgis, I.L. A comparison of the virulence of nine strains of axenically cultivated *Entamoeba histolytica* in hamster liver. *Arch. Invest. Med.* (Mex.) 5: Supl. 2: 423, 1974.

Diamond, L.S., Phillips, B.P. and Bartgis, I.L. The clawed jird (*Meriones unguiculatus*) as an experimental animal for the study of hepatic amebiasis. *Arch. Invest. Med*. (Mex.) 5: Supl. 2: 465, 1974.

Serial No. Z01 AI 00095-04 LPD
1. Parasitic Diseases
2. Parasite Growth and Diff. Sect.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemical basis for growth and differentiation of parasitic and free-living nematodes.

Previous Serial Number:

Principal Investigator: Dr. Ruth Pertel

Other Investigators: Dr. Carl F.T. Mattern, Dr. Lee Dobbs, Dr. Norman Luban, Nava Paran, Richard Hunzinger, Jr. and Deborah Olin

Cooperating Units: Laboratory of Viral Diseases, NIAID

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	6/6			6/6
Professional	6/6			6/6
Other				

Project Description:

Caenorhabditis elegans, a free-living nematode, was used as a model to study structure-function relationships during eukaryotic development. Specifically, the nervous system was singled out for study, since any behavioural changes which result from induced mutations serve as highly visible markers. In particular the structure and underlying biochemical properities of affected neurons were examined. In male nematodes the production of serotonin was correlated with particular neuronal cells of the tail. No serotonergic neurons were detected in the female (Luban and Pertel). Ultrastructure studies showed acetylcholinesterase to be localized in the neurons of the amphids (Pertel, Paran and Mattern), while studies with fluorescent labelled inhibitor probes showed acetylcholine receptors to be associated with sites on muscle and the amphids (Pertel).

Genetic mapping of temperature-sensitive developmental mutants showed that the same gene can regulate the timing of events in the egg and then at a later time become active and regulate other events when the gonads develop.

Ethyl methane sulfonate induced somatic cell mutations producing a variety of developmental abnormalities (terata). The response was dose related (Pertel and Olin).

The embryology of *Acrobelloides bütschlii* was worked out and the DNA characterized as to the guanine/cytosine base ratio and genome size (Pertel and Hunzinger).

This project is terminated.

Key Words:

Caenorhabditis elegans, free-living nematode, serotonin, acetylcholinesterase, temperature-sensitive developmental mutants, somatic cell mutations, genetics.

Publications:

Pertel, R., Paran, N., and Mattern, C.F.T. Ultrastructure localization of cholinesterase associated with the anterior structures of *Caenorhabditis elegans* (Nematode). *Exper. Parasitol*. In press.

Serial No. Z01 AI 00096-01 LPD
1. Parasitic Diseases
2. Parasite Growth and Diff. Sect.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Pathobiologic investigation of parasitic protozoa.

Previous Serial Number: None

Principal Investigator: Bruce P. Phillips

Other Investigators: Louis S. Diamond, Charles H. Zierdt, and Akio Takeuchi

Cooperating Units: Laboratory of Clinical Pathology, Clinical Center, NIH, and Laboratory of Experimental Pathology, Walter Reed Army Institute of Research, Washington, D.C.

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	12/12			12/12
Professional	12/12			12/12
Other				

Project Description:

Pathogenesis of enteric amebiasis was studied at ultrastructural level in gnotobiotic guinea pigs inoculated intracecally with enteric flora from patient with acute amebic colitis. Studies presented first documented information on mechanism of epithelial penetration by Entamoeba histolytica. Epithelial cells and PMN in close proximity to invading amebae showed severe cytoplasmic alterations whereas such degenerative changes were not evident in fibroblasts, smooth muscle cells, macrophages, lymphocytes, plasma cells, eosinophils, RBC, collagen fibers, and non-myelinated nerve bundles. Penetration by amebae of basal lamina was frequently accompanied by extrusion of epithelial cells into gut lumen resulting eventually in denuding the mucosa. Invasion of subepithelial tissue by amebae was accompanied by marked concentration of PMN in lamina propria suggesting chemotactic response of PMN to E. histolytica.

To ascertain why strains of E. histolytica vary to extreme in pathogenic potential and to identify factors responsible, numerous strains of E. histolytica of human derivation maintained by variety of in vitro procedures on different media, axenically and with different microbial associates, were evaluated for pathogenic potential by intracecal and/or intrahepatic inoculation into guinea pigs, hamsters, and jirds. Tissue from infected animals was compared histopathologically. Pathogenic strains of ameba maintained in vitro with mixed bacterial flora, wherein encystation and excystation occurred periodically, retained virulence for production of amebic enteritis. Same strains maintained axenically or monoxenically with bacteria or Trypanosomatidae did not produce cysts and lost ability to produce amebic enteritis.

Studies on primary amebic meningoencephalitis showed that when as few as 12 free-living amebae of the genus Naegleria were placed on the nasal mucosa of guinea pigs, they penetrated the mucosa into the arachnoid space and travelled to the cerebellum and cerebrum wherein they proliferated prodigiously destroying brain tissue with fatal termination. An intracellular symbiont isolated from the pathogenic strain of Naegleria is being used to infect non-pathogenic strains of the free-living ameba in search for a clue as to what triggers pathogenicity in this ubiquitous ameba.

To evaluate pathogenic potential of Blastocystis hominis, guinea pigs were inoculated orally, intracecally, and intrahepatically with the parastie derived from patients with severs diarrhea of unknown etiology. Approximately 40% of the animals inoculated orally and intracecally developed enteric B. hominis infection and some with the heaviest infections developed persistent diarrhea. Microscopic examination of cecal tissue from symptomatic animals showed B. hominis had penetrated the mucosa and was found in intraepithelial spaces. Epithelial penetration was unaccompanied by inflammatory response and amebae were never found in the lamina propria or beyond. Limited numbers of intrahepatic inoculations produced small hepatic "abscesses". These await histopathological evaluation.

Key Words:

Entamoeba histolytica, Amoeba, enteritis, amoebiasis, dysentery, pathogenesis, pathology, ultrastructure, epithelium, mucosa, virulence, hepatitis, axenic, primary amoebic meningoencephalitis, Naegleria, free-living, Blastocystis hominis, diarrhea, protozoa.

Publications:

Takeuchi, A., and Phillips, B.P. Electron microscope studies of experimental Entamoeba histolytica infection in the guinea pig. I.

Penetration of the intestinal epithelium by trophozoites. Am. J. Trop. Med. and Hyg. 24: 34, 1975.

Takeuchi, A., and Phillips, B.P. Electron microscopy of experimental amebiasis. Am. J. Path. 78: 54a, 1974.

Diamond, L.S., Phillips, B.P. and Bartgis, I.L. A comparison of the virulence of nine strains of axenically cultivated E. histolytica. Arch. de Investigacion Medica. In press.

Diamond, L.S., Phillips, B.P., and Bartgis, I.L. The clawed jird (Meriones unguiculata) as an experimental host for the study of hepatic amebiasis. Arch. de Investigacion Medica. In press.

Phillips, B.P. Naegleria: Another pathogenic ameba. Studies in Germfree guinea pigs. Am. J. Trop. Med. and Hyg. 23: 850, 1974.

Phillips, B.P., and Zierdt, C.H. Blastocystis hominis: Studies on its pathogenic potential in gnotobiotes. Exp. Parasitol. In press.

Serial No. Z01 AI 00097-17 LPD
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Pathological physiology and histochemistry of parasitic diseases.

Previous Serial Number: NIAID 123-B

Principal Investigator: Teresa I. Mercado

Other Investigators: None

Cooperating Units: Comparative Pathology Section, Division of Research Services (Dr. Anton M. Allen)

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	24/12			24/12
Professional	12/12			12/12
Other	12/12			12/12

Project Description:

Knowledge of the pathological physiology of parasitic diseases and of differences between parasitic strains is advanced by studying the susceptibility of host tissues to parasitic invasion and assessing the alteration of various physiological and histochemical parameters. Tissues from mice infected with *Trypanosoma cruzi* strains of different origins were processed histologically to assess parasitic invasion and pathologic changes. The extent of physiologic injury was determined by assaying the plasma and tissue isoenzymes of lactate dehydrogenase employing histochemical techniques, disc gel electrophoresis, planimetry, and densitometry. *T. cruzi* trypomastigotes were isolated from the blood of infected mice by ion-exchange chromatography. Studies of blood-induced and vector-derived infections with the Tulahuen (Chile), House 510 (Costa Rica), and House 11 (Nicaragua) strains, at various periods after inoculation, revealed the following: Increased plasma levels (3-4X) of total LDH activity in the Tulahuen and House 510 infections, but less than two times in the House 11 infections. The vector-derived infections elicited an increase in the total plasma LDH comparable to that of the blood-induced; however, some significant differences were observed, such as the higher level exhibited by the vector-derived Tulahuen-infected mice during the early period of infection. This finding was particularly relevant since parallel histochemical studies of such tissues as liver, heart, and skeletal muscle did not disclose significant pathologic changes. The Tulahuen strain, in the blood-induced infections, elicited the most rapid increase in plasma LDH; four times the normal value in four days. In all infections the increase of isoenzyme levels was progressive with length of infection. In addition to the five isoenzymes present in normal plasma (LDH-1, 2, 3, 4, 5), we observed one, more cathodic than LDH-5, identified by some investigators as LDH-X, a testicular isoenzyme. Some isoenzymes increased more significantly than others, such as LDH-4 and LDH-X, which in normal plasma occurred in minimal amounts. Increases to 10 and 39 times the normal level were observed in LDH-4 and LDH-X in the House 510 and Tulahuen strains respectively. In acute infections with these strains two additional isoenzymes, more cathodic than LDH-X were also observed. Studies on isoenzymes from homogenized liver, heart, and skeletal muscle, tissues which show extensive pathology in acute infections, revealed that the electrophoresed fractions generally were reduced in suspensions from liver and skeletal muscle. By contrast, amounts of heart isoenzymes were increased in some infected specimens, particularly LDH-5, which increased even when the total level had decreased. Trypomastigotes of _T. cruzi_ (Strain House 510), isolated from infected blood, exhibited one LDH isoenzyme which electrophoresed near LDH-5. Similarly, cultured epimastigotes (Tulahuen and House 510 strains) exhibited only one isoenzyme which migrated between LDH-2 and LDH-3. These findings indicate that the elevation of the plasma LDH isoenzymes was related to the pathologic change sustained by such tissues as liver, heart, and skeletal muscle, as demonstrated by the histochemical data. The influence of additional factors such as the involvement of other tissues and the parasite itself needs to be elucidated.

Basic cellular studies provide a means for understanding the physiological mechanisms involved in the response of the host to parasitic infections and may lead to the identification of specific parasite substances involved in pathogenesis. Histochemical studies aid in localizing the altered sites within tissues. Physiological studies quantitate the alteration of activity observed histochemically and aid in defining further the physiological mechanisms that may be damaged. Basic studies on strain differentiation aid in taxonomic classification and in assessing the pathogenicity of a parasitic infection.

Studies recently initiated employing more specific isoenzyme techniques (e.g., creatine phosphokinase, a muscle-specific enzyme) will be continued in an attempt to make a more definite distinction between strains. Studies with tissue LDH will be continued in order to assess further the differences observed during infection. Localization of the enzyme will also be visualized histochemically. Isoenzyme studies with _T. cruzi_ trypomastigotes will be continued.

continued.

Key words: Trypanosoma cruzi strains, histochemistry, pathologic physiology, lactate dehydrogenase isoenzymes, gel electrophoresis planimetry, densitometry, trypomastigotes, cultured epimastigotes, blood-induced infections, vector-derived infections.

Serial No. Z01 AI 00098-19 LPD
1. Parasitic Diseases
2. Physiology & Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemical mechanisms of energy metabolism in mammalian and parasitic organisms.

Previous Serial Number NIAID 123-C:

Principal Investigator: Eugene C. Weinbach

Other Investigators: Louis S. Diamond, Tsutomu Takeuchi (Visiting Fellow) and Dan Harlow (Staff Fellow).

Cooperating Units: Laboratory of Chemical Physics, NIAMDD (Hideo Kon)

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	24/12		12/12	36/12
Professional	12/12		12/12	24/12
Other	12/12			12/12

Project Description:

The objective of this project is to conduct fundamental studies on the mechanism of aerobic energy metabolism in mammalian and parasitic organisms. Currently, these studies center principally on mechanisms of electron transport and oxidative phosphorylation.

Tissues and cells are disrupted mechanically and subcellular fractions, chiefly mitochondria, isolated by differential centrifugation. Cellular and subcellular constituents, enzymatic activities and metabolic pathways are determined, characterized, and elucidated by enzymatic, chemical, radiochemical and physical methods. Oxygen consumption is determined polarographically with the Clark oxygen electrode.

Parasite studies: Continued investigation of the respiratory chain in *Entameba histolytica*, strain HK-9, cultivated axenically in Diamond's liquid medium, demonstrated unequivocally for the first time that iron-sulfur electron transferring proteins are functional in this parasite. Evidence was adduced from three experimental approaches: (1) Respiration of the trophozoites was inhibited by metal-chelating agents. Inhibition was correlated with the capacity of the chelator to form metal coordination complexes with iron. (2) Analytical studies disclosed that 87% of the total iron content is non-heme iron, and 86% of the total sulfide is acid-labile. Low temperature difference spectra (obtained in collaboration with Harlow) confirmed our previous studies done at the University of Stockholm (with Nelson and Ernster) that cytochromes are absent in this parasite. (3) The intact trophozoites, when examined by low-temperature electron paramagnetic resonance spectroscopy by Dr. Kon, NIAMDD, exhibited the characteristic non-heme iron signal at g=1.94 under conditions where the iron is in the reduced state, thereby providing conclusive evidence that non-heme iron proteins are functional in E. histolytica.

On the basis of our previous observations on oxygen uptake of E. histolytica, we are elucidating the mechanism of L-serine oxidation (Takeuchi). L-serine is not oxidized directly but first converted to pyruvate by L-serine dehydratase, and subsequently metabolized by the pyruvate oxidase system. We detected at least two types of pyruvate oxidases. One is P_i-dependent; i.e., phosphorylating pyruvate oxidase. The other is CoA-dependent. Although phosphorylating pyruvate oxidase has been characterized in bacteria, the CoA-dependent enzyme is novel, and represents a new type of pyruvate oxidase, having unique catalytic and molecular properties. It catalyzes the formation of acetyl CoA from pyruvate and CoA in the presence of oxygen. The acetyl CoA produced reacts with L-serine, the initial substrate of this metabolic pathway, to form O-acetyl-serine, which in turn reacts with hydrogen sulfide to form L-cysteine and acetate, the final products. Acetyl phosphate, produced by phosphorylating pyruvate oxidase, reacts with ADP to form ATP and acetate as the final products of this complex metabolic sequence.

Mammalian studies: Work with mammalian tissues was restricted to use of rat liver mitochondria as a basis of comparison for determination of cytochromes by low-temperature spectrophotometry, and non-heme iron by electron paramagnetic resonance spectroscopy. These studies were necessary to insure that the methodology employed with parasites were optimal and reproducible.

Continued studies on energy metabolism of human blood platelets disclosed the presence of highly active diaporase activity. Polarographic and spectrophotometric techniques showed that sonicated platelets have the enzymatic capacity to oxidize both reduced pyridine nucleotides, NADH and NADPH, in the presence of appropriate electron acceptors (Vitamin K_3 and dichlorophenolindophenol). Use of inhibitors confirmed the presence of diaphorase in these preliminary experiments. We also observed that "out-dated" platelets obtained from the blood bank no longer respond to thrombin, but are suitable for some of the enzymatic studies (with Harlow).

This investigation which attempts to elucidate vital biochemical mechanisms common to all aerobic cells has obvious importance for biochemical research in general (as shown by numerous collaborative projects in which we have participated during past years), and provides a basis for understanding bioenergetic problems associated with parasites and parasitism.

The parasite studies will be continued with emphasis on the isolation, characterization and physiological role of iron-sulfur proteins in E. histolytica. Other biochemical studies of this parasite will focus on elucidating the complete pathway of L-serine metabolism. The mammalian work will be continued with the objective of completing earlier preliminary studies on the role of non-heme iron in oxidative phosphorylation. Studies of diaphorase activity associated with energy metabolism in human platelets will be intensified. It is anticipated that two collaborative studies, suspended during this year, will be resumed. These are (1) continued investigation of the respiratory chain of Staphylococcus aureus with Dr. Theodore, Laboratory of Microbiology, NIAID, and (2) renewed investigations of the interrelation of heme synthesis and respiration in liver with Dr. Tshudy, NCI.

Key words: energy metabolism, electron transport, phosphorylation, iron-sulfur proteins, L-serine dehydratase, pyruvate oxidase, diaphorase, mitochondria, Entamoeba histolytica, human platelets, oxygen electrode.

Publications:

Weinbach, E.C. and Garbus, J.: Oxidative phosphorylation in mitochondria from aged rats. Reprinted in Brain Structure and Aging, MSS Information Corp. New York, 1974, pp 121-135.

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Muenzer, J., Weinbach, E. C. and Wolfe, S. M.: Oxygen consumption of human blood platelets. I. Effect of thrombin. Biochim. Acta 376: 237-242, 1975.

Muenzer, J., Weinbach, E.C. and Wolfe, S.M.: Oxygen consumption of human blood platelets. II. Effect of inhibitors on thrombin-induced oxygen burst. Biochim. Biophys. Acta 376: 243-248, 1975.

Bonkowsky, H. L., Tschudy, D. P., Weinbach, E. C., Ebert, P. S. and Doherty, J. M.: Prophyrin synthesis and mitochondrial respiration in acute intermittent porphria: studies using cultured human fibroblasts. J. Lab. Clin. Med. 85: 93-102, 1975.

Harlow, D. R., Weinbach, E. C. and Diamond, L. S.: Nicotinamide nucleotide transhydrogenase in Entamoeba histolytica, a protozoan lacking mitochondria. Comp. Biochem. Physiol. (In press).

Serial No. Z01 AI 00099-05 LPD
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biophysical Parasitology

Previous Serial Number: 123-E

Principal Investigator: James A. Dvorak

Other Investigators: None

Cooperating Units: Biomedical Engineering and Instrumentation Branch, DRS; Television Engineering Section, CC; Malaria Section, LPD; Division of Cancer Biology, Laboratory of Biochemistry, NCI.

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	24/12			24/12
Professional	12/12			12/12
Other	12/12			12/12

Project Description:

The major objective of this program is to develop and utilize biophysical techniques for the study of parasitological problems of medical importance.

The controlled-environment culture system and several electro-optical microscopy systems developed with this program are used to study host-parasite interactions at the cellular and subcellular levels.

(1) The interaction of T. cruzi with vertebrate cells.

(a) Drug Studies

The effects of Lampit on the intracellular cycle have been quantified. Lampit concentrations of less than 10^{-7}M have no effect on the intracellular cycle. At 10^{-6}M Lampit, the initial reproduction time is lengthened. Thereafter, the reproduction time increases linearly with increasing time of exposure to drug. Consequently, viable parasites remain in the host cells for long periods of time even when continuously exposed to 10^{-6}M Lampit. If drug pressure is removed, the parasites resume a normal reproduction time and complete the intracellular cycle. Continuous exposure to 10^{-5}M Lampit kills intracellular parasites that had no been previously exposed to drug. However, parasites can be made resistant to 10^{-5}M Lampit by rapid serial passage in vertebrate cells grown in increasingly higher concentrations of drug. The acquired drug resistance is stable; resistant parasites can be passaged in drug-free medium without loss of drug resistance.

(b) Attraction and accumulation of parasites to vertebrate cells.

Electro-optical instrumentation has been developed to quantify the attraction of trypomastigotes to vertebrate cells. Th attraction of a "myotropic" strain of parasites to skeletal muscle cells is about 2-fold greater than to HeLa cells and about 10-fold greater than to Vero (green monkey kidney) cells. Heat-inactivated skeletal muscle cells not only do not attract parasites, they have a significant repellant effect. However, trypomastigotes are capable of penetrating heat-inactivated cells and transforming to amastigotes. The kinetics of penetration of vertebrate cells is significantly different from the kinetics of attraction. Therefore, there are at least two interrelated parameters that modulate the initial phase of the infection.

One reason for the negative binomial distribution of a population of parasites among a population of host cells may be a natural "resistance" to invasion of certain cell types and an enhanced susceptibility of others. This is inferred from an analysis of the kinetics of "accumulation" of parasites in individual cells. The rates of accumulation are highly variable and zero rates are present under conditions where the probability of infection should be high. Resistance of susceptibility to infection could be due to factors such as genetic heterogeniety of the host cell population or natural differences in host cell membranes relative to the status of the cell in its life cycle.

(2) Defined medium cell culture of obligate intracellular parasites.

Trypanosoma _cruzi_ and _Toxoplasma_ _gondii_ have been grown for nearly one year in vertebrate cell culture using serum-free tissue culture medium. Qualitatively, the growth kinetics of _T_. _gondii_ appear to be the same as parasites grown in serum-supplemented cell culture. The RH strain of _T_. _gondii_ is still pathogenic to mice after 8 months of continuous culture in serum-free cell culture at inoculum levels equivalent to peritoneal-derived parasites. The intracellular cycle of _T_. _cruzi_ is qualitatively not the same in serum-free tissue culture as in a serum-supplemented system. The cycle appears lengthened and premature rupture of host cells may occur as judged by parasite morphology; more broad trypomastigotes and amastigotes are present in the extracellular fluid.

(3) Video microscopy.

Low-light-level video techniques have been developed to study the fluorescence emission of living cells in vitro. Short pulses of short wavelength, low-amplitude light are used for excitation. This protocol is required to prevent radiation damage to the specimen. The resulting reflected fluorescence is collected with high gain photon detectors and transmitted to a video storage tube where it can be scanned and recorded. Although the technique is limited by rather low optical resolution and high noise levels, both of these problems can be corrected. However, even in its present state the system is capable of visualizing the presence of naturally occuring autofluorescent compounds.

The video integrator described in a previous report is being used successfully to study the kinetics of spontaneous neoplastic transformation of vertebrate cells in vitro. Histograms and statistical analysis show a clear transition in cell parameters that were not analyzable prior to the introduction of the video integrator technique.

(4) Malaria research

Close collaboration between this laboratory and the Malaria Section continues in an effort to define basic cellular aspects of malaria (see report of L. H. Miller for details).

(1) The study of host-parasite interactions at the cellular and subcellular level is a topic of critical importance. The outcome of this interaction directly influences the physiologic state and survival potential of both the host and the parasite.

(2) The methodology and research philosophy being developed are directly applicable to other areas of biomedical research. For example, the controlled-environment culture system developed with this program is now in routine use in laboratories with such diverse interests as oncology, immunology, neurophysiology and morphogenesis. The video systems developed with this program are being used for analysis of macroscopic as well as microscopic images in basic research and diagnostic clinical-medical fields.

Research will continue in an attempt to further our understanding of the interaction of T. cruzi with vertebrate cells in vitro and relate this information to the course of Chagas' disease in nature. Basic information obtained with the controlled-environment culture system and video systems will be utilized in more complex in vitro model systems.

The basic methodology and research philosophy developed with this program will be used for the analysis of other host-parasite interactions. Most specifically, studies of the interaction between malaria merozoites and erythrocytes will be continued and preliminary work on an in vitro model for the exoerythrocytic stage of the cycle will be started.

Key words: tissue culture system, intracellular parasitism, video microscopy, Chagas' disease, malaria, toxoplasmosis.

Publications:

Luban, N. A. and Dvorak, J. A.: *Trypanosoma cruzi*: Interaction with vertebrate cells *in vitro*. III. Selection for biological characteristics following intracellular passage. *Exp. Parasitol.* *36*: 143-149 (1974).

Dvorak, J. A. and Poore, C. M.: *Trypanosoma cruzi*: Interaction with vertebrate cells *in vitro*. IV. Environmental temperature effects. *Exp. Parasitol.* *36*: 150-157 (1974).

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Dvorak, J. A., Miller, L. H., Whitehouse, W. C. and Shiroishi, T.: Invasion of erythrocytes by malaria merozoites. *Science* *187*: 748-750 (1975).

Miller, L. H., Shiroishi, T. and Dvorak, J. A.: Enzymatic modification of the erythrocyte membrane surface and its effect on malaria merozoite invasion. *J. Mol. Med.* (in press).

Miller, L. H., Aikawa, M., and Dvorak, J. A.: Malaria *(Plasmodium knowlesi)* merozoites: Immunity and the surface coat. *J. Immunol.* (in press).

Dvorak, J. A.: A new *in vitro* approach to quantitative studies of *Trypanosoma cruzi*. *Proceeding of the International Symposium on New Approaches in American Trypanosomiasis* (in press).

Miller, L. H., Mason, S. J., Dvorak, J. A., McGinniss, M. H., and Rothman, I. K.: Erythrocyte receptors for *(Plasmodium knowlesi)* malaria: The Duffy blood group determinants. *Science* (in press).

Serial No. Z01 AI 00100-03 LPD
1. Parasitic Diseases
2. Physiology & Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Terminal Electron Transport in Parasites

Previous Serial No.: 123-F

Principal Investigator: Dan R. Harlow

Other Investigators: Eugene C. Weinbach, Louis S. Diamond, Steven J. Mason and Louis H. Miller

Cooperating Units: None

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	12/12			12/12
Professional	12/12			12/12
Other	0			0

Project Description:

(1) The spectrophotometric study of cestode mitochondria cytochromes (mitochondria from Hymenolepis diminuta) has been completed with the aid of the Aminco DW-2 Dual-wavelength Spectrophotometer purchased last year. Recently, the method for preparing and quantitating the pyridine hemochromogen derivatives of cytochromes has been adapted for use in this laboratory. The pyridine hemochromogens of H. diminuta mitochondria will be determined and compared to those from rat liver mitochondria which we have already determined. An unusual situation occurs in the cestode mitochondrion in that only a cytochrome b was detected with an α-absorbtion band at 558 nm (cytochromes a and c which are present in other animal cell mitochondria are absent). This tentative conclusion will be tested by the pyridine hemochromogen study.

(2) A study of malarial parasite cytochromes has been initiated with Drs. Mason and Miller. Work to date has centered on finding a method for

removing hemoglobin from the erythrocyte ghost which is the major contaminant of the malarial parasite preparation. Sodium nitrite oxidizes the hemoglobin but should not affect the cytochromes; treatment of rat liver mitochondria by this method will determine any effect on cytochromes. Following oxidation of the interferring hemoglobin, the cytochromes in the malarial parasite preparation will be determined.

(3) Initial studies of the transhydrogenase of Entamoeba histolytica were completed and the paper accepted for publication. When the present demands on axenic amoeba decrease, it is anticipated that identification of the particle in the amoeba containing the transhydrogenase will be pursued.

(4) In collaboration with Dr. Weinbach, a spectrophotometric study of human platelet diaphorase has been initiated. Diaphorase, a terminal electron transport enzyme distinct from mitochondrial cytochromes, is of interest because of its possible connection with the cyanide-insensitive oxygen uptake burst seen in platelets after stimulation by thrombin. Previous work in this laboratory has established the presence of diaphorase in axenized E. histolytica. The study of the platelet diaphorase is pursued on its own merit as well as on its possible contribution to the understanding of the amoebal enzyme.

Parasites characteristically exhibit an anaerobic metabolism. This generally accepted dogma is confused by the fact that many parasites consume oxygen when it is available. Resolution of these conflicting observations has not been possible since the terminal electron transport mechanisms of parasites (e.g., presence or absence of mammalian-type cytochromes) are not known. It is of primary importance to determine the means by which parasites perform these vital biological oxidation-reductions both from the aspect of possible chemotherapeutic value, and from the aspect of intrinsic scientific merit.

Key Words:

electron transport, cytochromes, diaphorase, transhydrogenase, low temperature spectrophotometry, dual-wavelength spectrophotometry, Entamoeba histolytica, Hymenolepis diminuta, malarial parasites, human platelets.

Publications:

Harlow, D. R., Weinbach, E. C. and Diamond, L. S.: Nicotinamide nucleotide transhydrogenase in Entamoeba histolytica, a protozoan lacking mitochondria. Comp. Biochem. Physiol. (In press).

Serial No. Z01 AI 00101-01 LPD
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Protein synthesis and polysaccharide metabolism in the parasitic family trypanosomatidae, including *Trypanosoma cruzi.*

Previous Serial Number: None

Principal Investigator: Michael Gottlieb

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	9/9			9/9
Professional	9/9			9/9
Other	0			0

Project Description:

The objectives of this project are (1) To characterize protein synthesis in these parasites: in particular to distinguish between mitochondrial and cytoplasmic activities in an attempt to correlate such synthetic activities with stages in the life cycle. (2) To isolate and characterize polysaccharides in these parasites and to determine their subcellular location.

Organisms, insect trypanosomatids of the genus *Crithidia* and epimastigote (culture) forms of *Trypanosoma cruzi*, are grown on monophasic liquid medium and harvested according to standard techniques. The trypomastigote form of *T. cruzi* are obtained from infected tissue culture cells and is provided by A. Gam.

Protein synthesis is determined by incorporation of radiolabeled amino acid into hot acid insoluble material from whole cells and purified fractions.

Mitochondrial and Cytoplasmic Protein Synthetic Activities will initially be differentiated by use of antibiotics (chloramphenicol and cycloheximide). Subsequently, the enzymatic components (ribosomes and soluble factors) will be isolated and characterized by appropriate technics including gradient analysis and gel electrophoresis. Attempts will be made to characterize these components in both epi- and trypomastigote forms of T. cruzi.

Polysaccharide is purified from organisms by a phenol extraction of whole cells or fractions; and further purified by either ion exchange chromatography or enzymatic digestion of contaminating components. The polysaccharide is characterized as to monomer composition and size by chromatography and gradient analysis. The polysaccharide is characterized as to subcellular location (in particular its relationship to the cell surface) and its function.

As these investigations were initiated with the arrival of the investigator in October 1974 and represent a departure from preexisting approaches in the laboratory the findings to date are preliminary.

Protein Synthesis. Sucrose gradient techniques for the characterization of ribosomal components have been implemented. Techniques have also been implemented for incorporation of radiolabel into protein.

Polysaccharide. The polysaccharide component of the insect trypanosomatid Crithdia fasciculata has been isolated and partially characterized. The polysaccharide is composed to a large extent, if not entirely, of mannose. The polysaccharide sediments in sucrose gradients at 10-12s and from this and gel exclusion properties a molecular weight of approximately 200,000 may be estimated. The polysaccharide was labeled in vivo by growing cells in the presence of ^{14}C mannose and ^{14}C glucose. Further studies with labeled polysaccharide confirmed that this material is not an energy reserve, since it is not consumed under starvation conditions.

As has been obvious from many other systems an understanding of protein synthesis leads to a better understanding of cellular regulatory phenomenon. Members of the genus Trypanosoma undergo a developmental cycle during their existence in vertebrate and vertebrate hosts which must represent a differential expression of the total genome. These parasites contain an unusually high proportion of extranuclear DNA (i.e., localized in the hinetoplast region of the mitochondrion) and the alternate translation of this material and its control must contribute to the changes observed in the life cycle.

As with the protein synthesis aspects discussed above, studies on the polysaccharide material are of a fundamental nature. The role of this material is totally unknown, and a better understanding of it may contribute to our knowledge of cell function and architecture.

Studies along both lines, i.e., protein synthesis and polysaccharide metabolism, can proceed now that certain basic procedures have been worked

Mitochondrial and Cytoplasmic Protein Synthetic Activities will initially be differentiated by use of antibiotics (chloramphenicol and cycloheximide). Subsequently, the enzymatic components (ribosomes and soluble factors) will be isolated and characterized by appropriate technics including gradient analysis and gel electrophoresis. Attempts will be made to characterize these components in both epi- and trypomastigote forms of T. cruzi.

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Polysaccharide. The polysaccharide component of the insect trypanosomatid Crithdia fasciculata has been isolated and partially characterized. The polysaccharide is composed to a large extent, if not entirely, of mannose. The polysaccharide sediments in sucrose gradients at 10-125 and from this and gel exclusion properties a molecular weight of approximately 200,000 may be estimated. The polysaccharide was labeled in vivo by growing cells in the presence of ^{14}C mannose and ^{14}C glucose. Further studies with labeled polysaccharide confirmed that this material is not an energy reserve, since it is not consumed under starvation conditions.

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As with the protein synthesis aspects discussed above, studies on the polysaccharide material are of a fundamental nature. The role of this material is totally unknown, and a better understanding of it may contribute to our knowledge of cell function and architecture.

Studies along both lines, i.e., protein synthesis and polysaccharide metabolism, can proceed now that certain basic procedures have been worked out. During the course of the work, cell fractions will be examined for composition by electron micorscopy in collaboration with Dr. H. G. Sheffield.

With regard to the polysaccharide studies, its role in the cell may lead to work on the biochemical and immunological nature of the cell surface. Further studies will be made to examine the biosynthesis of the polysaccharide.

Key words: Trypanosomatidae, *Crithdia fasciculata*, *Trypanosoma cruzi*, biochemistry, protein synthesis, polysaccharides, mannose, metabolism, ribosomes, biosynthesis.

Publications: None

Serial No. Z01 AI 00102-01 LPD
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Pathogenesis of disease caused by infection with intracellular parasites.

Previous Serial Numbers: NIAID 125-B, NIAID 125-C and NIAID 125-D

Principal Investigator: Franklin A. Neva

Other Investigators: Bjarne Bjorvatn (Guest Woker), Richard Raizman (Associate), Jonathan Weintraub (Associate), and Albert Gam

Cooperating Units: None

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	27/12	23/12		50/12
Professional	5/12	23/12		28/12
Other	22/12			22/12

Project Description:

A model of leishmanial infection with an African strain of *L. tropica* has been established in a genetic in-bred strain of mice. Dose-response relationships, course of the disease and reproducibility of results have been determined and techniques were developed for harvesting intracellular parasites (amastigotes) for use in *in vitro* experiments with macrophages. One other strain of *L. tropica* failed to produce lesions in the in-bred mouse and additional strains are being studied. Factors that govern the response of different genetic stocks of mice to leishmanial infection are being sought by study of infections *in vivo* as well as by direct *in vitro* procedures involving parasite-cell interaction.

The coccidian organism from a cat discovered by Dr. Wallace of Pacific Research Section, which was originally thought to be a Sarcocyst (WC-1170) is now felt to represent a variety of toxoplasma organism of sufficient difference to warrant a new species designation (proposed Hammondia hammondi). Our attempts to obtain sustained in vitro multiplication of intracellular trophozoites in various cell cultures, using excysted sporozoites as inoculum, were unsuccessful. However, it appeared that some limited initial multiplication occurred in some experiments. We have also confirmed Wallace's findings that mice inoculated either orally or parenterally with oocysts may develop a low level antibody response to T. gondii trophozoites and that the tissues of mice are infective when fed to cats. The possible significance of this close relative of the usual toxoplasma organism as far as human infection and disease might be concerned remains to be determined.

Further experience has been gained with the circulating antigen that can be detected in plasma of toxoplasma infected animals. Antibody against the circulating toxoplasma antigen is prepared in rabbits and this antibody can be linked to activated sepharose to prepare an affinity column for concentrating antigen. Material suspected of containing toxoplasma antigen can then be examined for its presence by counter current immunoelectrophoresis. Questionably positive reactions for antigen have been obtained with several sera from patients, but a more sensitive test will probably be required for clinical specimens. Preliminary results with a solid-phase radioimmunoassay procedure are reasonably encouraging that circulating toxoplasma antigen may be detected with this test.

Efforts have continued to further characterize and purify antigens from T. cruzi grown in cell cultures. In antigens tested by complement-fixation (CF) a cross-reacting component is frequently present which reacts with sera from patients with leishmaniasis. Other evidence indicates that the cross-reacting component is derived from host cells in which T. cruzi is grown. Both the specific and cross-reacting components were recently found to be eluted in the same fraction by Sephedex chromatography. In contrast to CF, the cell culture antigen shows no cross-reactivity by gel diffusion. The importance of specific and sensitive diagnostic antigens for study of T. cruzi infections is underscored by our recent finding of a small group of presumed positive reactors among over seven hundred sera from a survey in Texas. We hope to follow up some of these cases to re-check serology and examine for evidence of disease.

Key Words:

Leishmania, coccidia, toxoplasma, T. cruzi, antigens, chromatography, gel-diffusion, immunoelectrophoresis, radioimmunoassay, genetic factors.

Publications:

Glew, R.H., Briesch, P.E., Krotoski, W.A., Contacos, P.G., and Neva, F.A. Multidrug-resistant strain of P. falciparum from Eastern Colombia. J. of. Infect. Diseases. 129: 385, 1974.

Miller, L.H., Glew, R.H., Wyler, D.J., Howard, W.A., Collins, W.E., Contacos, P.G., and Neva, F.A. Evaluation of clindamycin in combination with quinine against multidrug-resistant strains of *P. faciparum*. *Am. J. Trop. Med. & Hyg*. *23*: 565, 1974.

Cicmanec, J.L., Neva, F.A., McClure, H.M., and Loeb, W.F. Accidental infection laboratory-reared *Macaca mulatta* with *T. cruzi*. *Lab. Animal Science*. *24*: 783, 1974.

Glew, R.H., Atkinson, J.P., Frank, M.M., Collins, W.E., and Neva, F.A. Serum complement and immunity in experimental simian malaria. I. Cyclical alterations in C4 related to schizont rupture. *J. Infect. Dis*. *131*: 17, 1975.

Atkinson, J.P., Glew, R.H., Neva, F.A., and Frank, M.M. Serum complement and immunity in experimental simian malaria. II. Preferential activation of early components and failure of depletion of late components to inhibit protective immunity. *J. Infect. Dis*. *131*: 26, 1975.

Raizman, R.E. and Neva, F.A. Detection of circulating antigen in acute toxoplasma infection of experimental animals. *J. Infect. Dis*. In press.

Serial No. Z01 AI 00103-08 LPD
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Immunological studies on toxoplasmosis and other parasitic diseases.

Previous Serial Number: 125-P

Principal Investigator: Milford N. Lunde

Other Investigators: Louis S. Diamond, Kendall G. Powers, Harley Sheffield

Cooperating Units: None

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	19/12			19/12
Professional	14/12			14/12
Other	5/12			5/12

Project Description:

The search for improved sero-diagnostic tests and attempts to better understand the immune factors involved in host parasite relationship require new and better antigens.

Several methods of preparing antigen for the malaria indirect hemagglutination test, IHA, were investigated. Lysis of erythrocytes with saponin removal of the hemoglobin containing supernatant by centrifugation followed by freeze-thawing of the resuspended plasmodium was found to be the most efficient method of obtaining antigen. Evaluation of malaria antigens and sero-diagnostic tests is being done in collaboration with the Section of Malaria (LPD) and in cooperation with European workers.

In intestinal infections such as cholera IgA antibodies in the intestinal mucosa have been shown by others to play a protective role. Using the indirect fluorescent antibody test, IFAT, for Entamoeba histolytica specific

IgA antibody has been demonstrated in sera obtained from three patients with acute amebic dysentery. No or very little E. histolytica specific IgA antibody was found in the serum from patients with amebic liver abcess. IgM specific antibody, however, was found up to titers of 1:80 in the serum from patients with amebic liver abcesses.

Previous attempts to purify antigen for the sero-diagnosis of amebiasis by means of Sephadex G-200 have been unsuccessful. Purification has now been obtained using an immunoadsorbent column to which globulin from a patient with an amebic liver abcess has been bound. This procedure needs additional refinement to increase its efficiency and hopefully it can be adapted for the purification of other parasite antigens.

Key words: Toxoplasma, amebiasis, malaria, antigens, antibody.

Publication:

Collins, W.E., Lunde, M.N., and Skinner, J.C.: Development of antibodies to Plasmodium vivax as measured by two different serologic techniques. Am. J. Trop. Med. Hyg. (In press)

Serial No. Z01 AI 00104-01 LPD
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Structure and development of parasitic protozoa

Previous Serial No.: 125A and 125I

Principle Investigator: Harley G. Sheffield

Other Investigators: Marjorie L. Melton

Cooperating Units: Animal Parasitology Institute, ARS-USDA, Beltsville, Maryland. Department of Entomology, University of Maryland, College Park, Maryland.

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	36/12			36/12
Professional	24/12			24/12
Other	12/12			12/12

Project Description:

Cellular structure and changes related to growth, differentiation and multiplication of parasitic protozoa and response of host tissues to parasitic infection are studied. Aspects of infection and disease related to structure and development, particularly in toxoplasmosis, are examined.

Immunological response in relation to prevention of reinfection is studied in toxoplasma-infected cats. In addition to standard parasitological techniques, in vitro cultivation and electron microscopy are utilized.

Attempts to actively immunize cats against oocyst-producing infections by Toxoplasma gondii have been partially successful. Cats treated with a new anticoccidial drug, lasolacid, can be infected with T. gondii and circulating antibody can be detected. However, oocysts, which are infective to man, are not excreted by the cat. Subsequent challenge with T. gondii results in slight elevations of antibody titer but no oocysts are produced which indicates that the cats are resistant. Some cats excreted oocysts in the initial infection following completion of the treatment regimen. However, these failures are believed to be related to inadequate drug levels and further experiments using higher dosages are planned.

Development of a technique to eliminate production of toxoplasma oocysts by cats is important since the cat is a potential reservoir of human toxoplasmosis.

Electron microscopic studies of Sarcocystis fusiformis in dogs are being continued in collaboration with the Animal Parasitology Institute. Late sexual stages and oocysts (sporocysts?) were observed. Structural differences between these stages and those of Toxoplasma and eimerian species of coccidia were noted and indicate that Sarcocystis is closely related but taxonomically different from the latter parasites.

The relationship between Sarcocystis fusiformis, Toxoplasma gondii and other coccidia is biomedically important since one species of Sarcocystis has been implicated in the production of isosporosis in man.

Proposed studies include drug attenuation of toxoplasmosis in cats, ultrastructure of T. gondii development in vitro and in vivo and development of Sarcocystis fusiformis in dogs. Ultrastructural aspects of in vitro development of Giardia will be examined when culture techniques are developed.

Key words: Toxoplasma, Sarcocystis, ultrastructure.

Publications:

Sheffield, H.G. and Melton, M.L.: Effect of pyrimethamine and sulfadiazine on the fine structure and multiplication of Toxoplasma gondii in cell cultures. J. Parasitol. In press.

Sheffield, H.G. and Melton, M.L.: Activity of the anticoccidial compound, lasolacid, against Toxoplasma gondii in cultured cells. J. Parasitol. In press.

Jones, J.C. and Sheffield, H.G.: The fine structure of mature adipocytes of adult female Aedes aegypti mosquitoes. J. Entomol. In press.

Serial No. Z01 AI 00105-03 LPD
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanism of Invasion of malarial merozoites.

Previous Serial Number: NIAID 132 A

Principal Investigators: Louis H. Miller, Steven J. Mason and James A. Dvorak

Other Investigators: Tsugiye Shiroishi, Ivan Rothman and Mary McGinnis

Cooperating Units: Institute of Pathology, Case Western Reserve University, Cleveland, Ohio; Walter Reed Army Institute of Research, Washington, D.C.; Blood Bank, Clinical Center, NIH.

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	36/12			36/12
Professional	36/12			36/12
Other				

Project Description:

To understand the mechanism by which the malarial merozoite gains entry into the erythrocyte and to evaluate methods for blocking invasion.

Schizont infected erythrocytes of Plasmodium knowlesi were cultured so that merozoites would be released. Studies included direct observation of merozoite-erythrocyte interaction, influence of alteration of the erythrocyte membrane on invasion, invasion of ghosts, immunity and the merozoite surface coat, and Duffy blood group determinants and susceptibility to invasion.

Direct observation of merozoite-erythrocyte interaction. The sequence of events in invasion has been well characterized. The widespread perturbation of the erythrocyte membrane after initial contact with a merozoite causes no evident irreversible change in the membrane. During localized endocytosis a lesion is produced in the membrane that increases local permeability to salt and water. If the endocytotic vacuole is not resealed, the erythrocyte swells and lyses. Spinning of merozoites can be observed in the vacuole during the rapid swelling of the erythrocyte. At the time of lysis, the spinning stops. The parasite initiators of perturbation and endocytosis are unknown.

Influence of membrane alteration on invasion. Chymotrypsin or pronase treatment of human erythrocytes blocked invasion by malarial (*Plasmodium knowlesi*) merozoites. The concentration of enzymes that blocked invasion (0.1 mg/ml or greater) was the same as that which reduced protein band III and acetyl cholinesterase activity of the surface. Lower concentrations did not block invasion or have proteolytic activity. Proteolytic enzymes failed to block invasion of monkey erythrocytes. Despite this, digestion of protein band III and acetylcholinesterase by chymotrypsin was similar for human and rhesus erythrocyte. The data suggest that the receptor for *P. knowlesi* on human erythrocytes is a glycoprotein and on rhesus erythrocyte, a glycolipid.

Invasion of ghosts by merozoites. In order to study the importance of various erythrocyte components for invasion by merozoites, resealed erythrocyte ghosts with different intracellular compositions were prepared and their interaction with merozoites observed. The widespread deformation of the erythrocyte membrane upon attachment by a merozoite, previously observed with intact red cells, was seen with ghosts containing both high and low levels of ATP. Only ghosts with high ATP concentrations, however, were invaded.

Immunity and the merozoite surface coat. Merozoites that were agglutinated by immune sera were still able to attach to and deform erythrocytes but usually were unable to invade. Marked merozoite aggregation was usually associated with reduced invasion of erythrocytes in culture. The agglutination and reduced invasion were immunologically specific. A surface coat appeared on merozoites after release from erythrocytes in medium with or without fetal calf serum added. The coat was susceptible to trypsin treatment. The coat was agglutinated by immune serum and may be the functional antigen in merozoite immunization.

Duffy blood group determinants and susceptibility to *P. knowlesi* merozoites. Duffy blood group negative human erythrocyte (FyFy) are resistant to infection by *Plasmodium knowlesi*, a simian malaria that infects Duffy positive human erythrocytes. Removal of the Duffy blood group determinants on human erythrocytes by proteolytic enzymes or blocking the receptors with anti-Duffy serum markedly reduced invasion by knowlesi merozoites. Duffy blood group determinants were found on rhesus monkey erythrocytes. Proteolytic digestion that had no influence on invasion of rhesus erythrocytes also did not reduce their Duffy reactivity.

The P. knowlesi resistance factor, Duffy negative erythrocytes, occurs in high frequency in West Africa, where the people are resistant to vivax malaria. This suggests that Duffy blood group determinants (Fy^a or Fy^b) may be erythrocyte receptors for P. vivax.

The identification of the receptor on the erythrocyte membrane might permit isolation of the components on the merozoite that initiate invasion. It may then be possible to immunize the host against this site on the merozoite in order to block erythrocyte penetration.

We now plan to isolate the Duffy blood group determinant and characterize its structure, insert the Duffy determinant into nonsusceptible cells and test susceptibility, study the relation between Duffy negative erythrocytes and P. vivax in blacks, isolate merozoites for immunization studies, and study properties of the erythrocyte membrane that are necessary for invasion.

Key Words:

Merozoite, Duffy blood group determinant, malarial receptors, erythrocyte invasion, malaria surface coat, erythrocyte ghosts.

Publications:

Fremount, H.N., and Miller, L.H. Deep vascular schizogony in Plasmodium fragile: Organ distribution and ultrastracture of red cells adherent to vascular endothelium. Am. J. Trop. Med. Hyg. 24: 1, 1975.

Dvorak, J.A., Miller, L.H., Whitehouse, W.C., and Shiroishi, T. Invasion of erythrocytes by malaria merozoites. Science. 187: 748, 1975.

Aikawa, M., Miller, L.H. and Rabbege, J. Erythrocyte membrane alteration in Plasmodium vivax and Plasmodium cynomolgi by electron microscopy and its possible relation to Schuffner's dots. American J. Pathology. In press.

Miller, L.H., Aikawa, M., and Dvorak, J.A. Malaria (Plasmodium knowlesi) merozoites: Immunity and the surface coat. J. Immunol. In press.

Miller, L.H. Treatment of malaria. In Conn, H.A. (Ed.): Current Therapy. In press.

Dvorak, J.A., Miller, L.H., Whitehouse, W.C. Movie: Invasion of erythrocytes by malarial merozoites.

Miller, L.H., Mason, S.J., Dvorak, J.A., Rothman, I. and McGinnis, M. Erythrocyte receptors for (P. knowlesi) malaria - The Duffy blood group determinants. Science. In press.

Herman, R., and Shiroshi, T. Plasmodium gallinaceum: Selective immunosuppression by cyclophosphamide in Preerythrocytic malaria. Exp. Parasitol. 34: 295, 1973.

Serial No. Z01 AI 00106-03 LPD
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on humoral immunity of malaria and other parasitic diseases.

Previous Serial Number: NIAID 132 B

Principal Investigator: Kendall G. Powers

Other Investigators: L.H. Miller and M. Lunde

Cooperating Units: None

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	13/12			14/12
Professional	8/12	1/12		9/12
Other	5/12			5/12

Project Description:

Studies are being conducted to identify and characterize different malarial antigens and their respective antibodies in order to determine their relative role in the overall immune response. Various host parasite models are used in these studies including Plasmodium knowlesi and P. cynomolgi in rhesus monkeys, P. falciparum in owl monkeys and P berghei and P. vinckei in rodents. Antigenic variation in malaria is being studied in vivo and in vitro using the schizont agglutination test to monitor antigenic differences between parasite populations during acute and chronic malarial infections. The effect of host immunity on the invasion of erythrocytes by merozoites is being investigated in an attempt to correlate antimerozoite activity with functional immunity to malaria.

We also are evaluating and comparing methods of preparing malarial antigens for the indirect hemagglutination test.

a) A micromethod for conducting the schizont agglutination test has been devised. Using this test it has been shown that malarial antibodies are detectible about 5 days after peak parasitemia in monkeys infected with blood-induced P. knowlesi (1:27). These antibody titers rapidly increase to 1:500,000 during the next 10 days and remain at this level for at least 8 months. During this period numerous recrudesences occur and when these recrudesced parasites are subinoculated into noninfected monkeys they were found to be antigenically different from the original inoculum. This variation in the antigenic character of the parasite strain might explain why it can multiply in an otherwise immune host. b) Monkeys were injected repeatedly with the same antigenic variant and then challenged with this variant or with two other strains of P. knowlesi. Serum before and after challenge was assayed for schizont agglutinating antibodies and antimerizoite antibody. There was a correlation between the presence of these antibodies and functional immunity as evidenced by delayed onset of infection, peak parasitemias and the outcome of the infection. c) The IHA test is now widely used for the surveillance of antibody to malaria. One of the problems in preparing the antigen is separating parasites from contaminating erythrocytes and hemoglobin. Ficoll density gradients were used to obtain purified preparations of schizont-infected erythrocyte and this was compared with a more simple method of saponin lysis, to remove uninfected cells and hemoglobin, followed by freeze-thaw to break up infected cells and release the soluble parasite antigen. Although any antigen in the parasitophorous vacuole would be lost in the saponin procedure this did not appear to influence the specificity of the preparation. In fact, preparation by the more simple saponization, freeze-thaw procedure yielded more malarial antigen than the ficoll method without loss of sensitivity and appears to be the most practical method of preparing antigen for the IHA test.

It is hoped that these studies will develop methods to classify antigenic varients within malaria populations, to separate and identify the antigens on the surface of the infected red cell and to identify the stage of the parasite responsible for malarial immunity. During the malarial infection, the host produces antibodies to infected red cells that are coated with malarial antigens. Probably of equal importance for functional immunity are the antigens present on the surface of merozoites. Circulating antibody, with or without complement, could effectively destroy the merozoite or block its infectivity to erythrocytes. If there are antigenic variants im malaria then the problem of immunization becomes more difficult, even if the immunogens can be identified.

Work on the application of the schizont agglutination test in studying malarial immunity will be continued. Other investigations will involve the identification and characterization of the antigenic components that are on the erythrocyte surface, within the red cell, between the membrane and the parasite and contained in the free parasite.

Key Words:

Humoral immunity, schizont agglutination test, Plasmodium knowlesi, antigenic variation, merozoite invasion.

Publications:

Abdel-Wahab, M., Powers, K.G., Mohmoud, S.S., and Good, W.C.: Suppression of schistosome granuloma formation by malaria in mice. Am. J. Trop. Med. Hyg. 23: 915, 1974.

Warren, M., Powers, K.G., Garnham, P.C.C., and Shiroishi, T. Plasmodium cynomolgi: Influence of x-irradiation and sporozoite dilution on relapse patterns in infected rhesus monkeys. Expt. Parasit. 35: 266, 1974.

Serial No. Z01 AI 00107-04 LPD
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Chemotherapy of malaria and other protozoan diseases

Previous Serial Number: NIAID 132 C and NIAID 132 D

Principal Investigators: Richard L. Jacobs and Kendall G. Powers

Other Investigators: Masamichi Aikawa and Louis C. Koontz

Cooperating Units: Case Western Reserve University

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	24/12			24/12
Professional	11/12			11/12
Other	13/12			13/12

Project Description:

The purpose of this project is to evaluate the activity of various drugs against blood and tissue stages of malaria in animal models including Plasmodium berghei and P. vinckei in mice, P. knowlesi and P. cynomolgi in rhesus monkeys and P. falciparum in owl monkeys. Compounds also are evaluated against strains of malaria resistant to chloroquine, pyrimethamine, quinine, DDS, or other drugs. The mechanisms of acquired drug resistance in malarial parasites are investigated by developing and characterizing different drug resistant strains of malarial parasites.

The development of a strain of P. berghei that is totally resistant to clindamycin has been described in a previous report. Using this resistant strain, comparison of clindamycin uptake by uninfected erythrocytes and erythrocytes parasitized with either clindamycin-sensitive or with clindamycin-resistant P. berghei have been made using ^{3}H-clindamycin both in vivo and in vitro. No significant differences in accumulation of ^{3}H-clindamycin have been detected between sensitive and clindamycin-resistant parasites utilizing both scintillation counting and autoradiography. Plasma drug levels were observed to rise and fall in accordance with erythrocyte levels. These results suggest that the development of resistance to clindamycin does not involve differences in uptake of the drug by resistant and sensitive parasites as in the case of resistance to chloroquine.

Mefloquine (WR 142490), a 4-quinolinemethanol compound, has recently been reported to have prolonged suppressive antimalarial action in man against strains of P. falciparum that are resistant to commonly used antimalarials, including chloroquine and pyrimethamine, when administered as a single oral dose. Mefloquine protects man against sporozoite-inoculated P. falciparum for periods up to two weeks following a single oral dose. Against P. berghei, a single oral dose of 50 to 200 mg/kg mefloquine is effective in temporarily clearing parasites from the blood and curing some mice. However, no prophylactic activity was detectable when mefloquine was administered either three or seven days prior to inoculation of blood forms of P. berghei. Mefloquine was completely ineffective against a quinine-resistant strain of P. berghei in mice.

The morphologic and clinical effects of clindamycin and N-demethyl-4'-pentyl clindamycin were evaluated against P. knowlesi in rhesus monkeys. Drug induced morphologic changes in the parasite were studies by light and electron microscopy. It was found that the primary site of action of these drugs was on the ribosomes with secondary effects, probably not directly related to drug action, on the mitochondria and nucleus. It appears that the mode of action of these antibiotics is different from the commonly used antimalarials. Both compounds cured blood-induced infections of P. knowlesi when given daily for 5 consecutive days. Although these compounds were able to control fulminating infections in all treated monkeys their effect was about 2 days clower that chloroquine in decreasing parasitemias and 3-4 days slower in clearing parasites from the blood. A single dose of clindamycin and the same effect as 5 daily doses in clearing parasites from the blood, however, several doses were required to cure the infection. Clindamycin also had an effect on the morphology of gametocytes and their infectivity to mosquitoes.

Certain strains of P. falciparum are resistant to chloroquine and other synthetic antimalarials and there exists need for a safe, efficacious drug for the treatment of this disease. Considerable effort has been expended to find such drugs and two of the more promising compounds in this respect are clindamycin and mefloquine. These drugs have been shown to possess a significant degree of antiparasitic activity against human malaria and may prove useful in the treatment of resistant strains of falciparum malaria. Also, other species of malaria that infect man may become refractory to presently effective drugs. The appearance of drug-resistance in malarial parasites of man has served to emphasize the need for a better understanding of the basic mechanisms of drug action on the parasite and other aspects of host-drug parasite relationships.

This study will be continued with emphasis on explaning the mechanism(s) by which parasites become resistant to drugs. Using radio-labelled clindamycin, differences in uptake of the drug between sensitive and resistant strains will be further investigated. As promising compounds become available they will be evaluated against normally sensitive and drug resistant strains of malaria.

Key Words:

Chemotherapy; malaria; clindamycin; mefloquine; drug resistance; mechanism of drug action; antimalarials.

Publications:

None

Serial No. Z01 AI 00108-04 LPD
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the biology and immunogenicity of malarial sporozoites

Previous Serial Number: None

Principal Investigator: David H. Chen and Robert W. Gwadz

Other Investigators: Dr. L.H. Miller, Dr. R.L. Jacobs and Dr. J.A. Dvorak

Cooperating Units: None

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	18/12			
Professional	16/12			
Other	2/12			

Project Description:

The purpose of this project is to examine the biology of the malarial sporozoite with emphasis on its mode of infection, potential for latency in relapsing malarias, and capacity as an immunogen. Plasmodium berghei in rodents, and P. knowlesi and P. cynomolgi in monkeys as well as several species of Anopheles mosquitoes are being used as models for these studies.

The mechanism of sporozoite infection of liver cells either directly or via macrophages is being studies in vitro with phase contract and interference microscopy.

Work is continuing on the course of sporozoite invasion in vivo using tritium labelled parasites. To date we have been unable to recover labelled sporozoites from either liver sections or from dissociated liver preparations.

The P. berghei rodent model of sporozoite-induced immunity is being investigated for the role of cellular immunity in protection. Furthermore, the requirements for attentuation and inactivation of sporozoites as immunogens are being examined.

Concurrently P. cynomolgi sporozoite preparations harvested from whole mosquitoes and subjected to density gradient centrifugation are being purified using various immuno/physical techniques. Sporozoites freed from extraneous material will be used in vivo and in vitro to determine where functional immunity can be demonstrated.

Immunization using X-irradiated P cynomolgi and P knowlesi sporozoites in several groups of Rhesus monkeys are now underway. The immune status of these animals is being monitored for the production of anti-sporozoite antibodies and later for cellular immunity by in vitro blastogenesis. The development of a monkey model for sporozoite immunity would provide a valuable tool for the study of immune phenomena in malaria and facilitate evaluation of this technique prior to field application.

Key Words:

Mosquito transmission, mechanisms of sporozoite invasion, sporozoite-induced immunity, P. berghei, P. knowlesi, P. cynomolgi, sporozoite as an immunogen.

Publications:

None

Serial No. Z01 AI 00109-03 LPD
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cell-mediated immunity in malaria

Previous Serial Number: NIAID 132 H and NIAID 132 G

Principal Investigator: David J. Wyler

Other Investigators: Louis H. Miller, John I. Gallin

Cooperating Units: Laboratory of Clinical Investigation, NIAID; Medical Research Council Laboratories, Fajara, The Gambia (West Africa); Southern Research Laboratories, Birmingham, Alabama.

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	24/12			24/12
Professional	12/12			12/12
Other	12/12			12/12

Project Description:

To study several aspects of cellular immunity in human and animal malaria infections with particular emphasis on the role of thymus-dependent lymphocytes (T cells) and of the spleen.

Human studies: Lymphocyte transformation *in vitro* to mitogens and malaria antigen was studied in adults and children in West Africa. Using rosette formation techniques, subpopulations of peripheral lymphocytes were quantitated during *P. falciparum* infections. Different approaches to the consideration of a B-cell mitogen have been and continue to be pursued.

Animal studies: The effect of splenectomy on the course of malaria in rhesus monkeys infected with *P. inui* and *P. cynomolgi* is being studied in a retrospective review of data (with Dr. Schmidt). The mechanism of splenomegaly in malaria and its relation to macrophage cytokinetics is studied in mice infected with *P. berghei*. In this regard, susceptibility of mice to malaria with a genetic defect in macrophage function, (Chediak Higashi Syndrome) has been studied. Preliminary susceptibility studies were also undertaken as a basis for future experiments to ascertain if immunity to malaria is genetically linked to the H2 locus in mice.

Adults and children in an endemic region have evidence of significant T cell sensitization to *P. falciparum*. Preliminary evidence suggests that a serum factor in children with malaria suppresses *in vitro* transformation of this lymphocyte to PHA, Con A and falciparum antigen. Total circulating T-cell number in malaria is depressed with a concomitant increase in null cells in many patients; treatment rapdily restores peripheral lymphocytes to a normal distribution. Circulating lymphoblasts in malaria have been observed and are being characterized. To date, no direct evidence for a B-cell mitogen in malaria has been found.

Monkeys infected with *P. inui* die if splenectomized before infection, but cure themselves of the infection if splenectomized after inoculation. In contrast, intact monkeys suffer recrudescences for up to 16 years. Spleens of mice with *P. berghei* infection but not controls contain a heat- and trypsin-sensitive substance of 80,000 MW which is present within four hours of the infection and attracts blood monocytes to the spleen. Monkeys with malaria also contain such a chemotactic factor (CTX). Chediak-Higashi mice, whose leukocytes have functional defects are not more susceptible to malaria than litter-mate controls.

Parts of this research have demonstrated that T cells in human malaria may play a critical role in host defence. Further study may provide insights necessary forming a basis for work in the immunoprophylaxis of malaria in man. Furthermore, the intravascular nature of malaria infection provides a unique model with which to investigate critical questions regarding the role of the spleen in infectious diseases generally.

Studies in lymphocyte transformation in malaria are being continued in collaboration with the M.R.C. Laboratories in The Gambia. These will attempt to solidify preliminary observations and initiate longitudinal studies in this patient populations. Experiments testing hypotheses generated by the splenectomy data will soon be instituted. Expansion of observations regarding spleen CTX elaboration and further purification of the substance is planned. Susceptibility studies in mice will be set up to test H2 gene linkage of immune response in malaria.

Because the principal investigator will be away from the N.I.H for one year (June 30, 1975-July 1, 1976) these projects will be temporarily curtailed.

Key Words:

Malaria, cell-mediated immunity, T-cells, spleen, chemotaxis, macrophages, immunosuppression, Chediak-Higashi Syndrome.

Publications:

Wyler, D.J. and Oppenheim, J.J. Lymphocyte transformation in human *Plasmodium* falciparum malaria. *J. Immunol.* *113*: 449, 1974.

Serial No. Z01 AI 00110-03 LPD
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on gametogenesis, gamete-specific immunity and the infectivity of malarias to mosquitoes.

Previous Serial Number: NIAID 133 G

Principal Investigators: Robert W. Gwadz and Richard Carter

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	12/12			12/12
Professional	12/12			12/12
Other				

Project Description:

The objectives of this project are: 1) to understand the biological and biochemical factors which regulate the maturation and exflagellation of malaria gametocytes; 2) to understand the mechanism by which the microgamete achieves fertilization and to determine factors enhancing or inhibiting fertilization; 3) to determine the influence of vertebrate host factors on exflagellation, fertilization and development of the malarial organism in the mosquito gut; 4) to develop a method for concentrating and freezing gametocytes without reducing their infectivity to mosquitoes.

With Plasmodium cynomolgi and P. knowlesi in monkeys, P. gallinaceum in chickens and P. berghei in rodents as model systems, an in vitro membrane blood-feeding system is being used to measure the effects of various chemical compounds and blood components on the infectivity of gametocytes to mosquitoes. Concurrently, the effects of these treatments on exflagellation and fertilization are being observed with phase contrast and interference microscopy.

To date we have shown that in vitro exflagellation of avian and mammalian malarial microgametocytes is reverseably inhibited by suspension in tris NaCl and glucose. Resuspension after up to several hours in a solution of bicarbonate, tris NaCl, glucose and bovine serum reinitiates normal exflagellation and infection of mosquitoes.

In studies concerning gamete-specific immunity we have found that serum from monkeys chronically infected with P. cynomolgi can reduce or eliminate oocyst development in mosquitoes. At the same time, normal serum can restore the infectivity of gametes inhibited by vertebrate host factors yet to be determined.

An understanding of the biology of the sexual cycle of the malaria parasite and "gamete-specific" immunity should provide insights into some of the basic mechanisms of host immunity and cycles of host infectivity. "Gamete-specific" immunization offers potential for interruption of malaria transmission in endemic areas. A method for gametocyte storage is a necessary prerequisite for planned studies of the genetics of drug resistance in malaria.

This project will continue and expand to include immunization of animals against gametes.

Kew Words:

Malaria, Plasmodium sp., gametogenesis, gametocyte, gamete, fertilization, exflagellation, immunization, immunity.

Serial No. Z01 AI 00111-03 LPD
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the genetics and susceptibility of anopheline mosquito vectors of human and simian malaria.

Previous Serial Number: NIAID 133 I

Principal Investigator: Robert W. Gwadz

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total	4/12			4/12
Professional	2/12			2/12
Other	2/12			2/12

Project Description:

This project was initiated at the Primate Malaria Unit of the LPD in Chamblee, Georgia and was temporarily suspended when the Unit was transferred to Bethesda. New insectary facilities are now complete and a technical staff has been trained so that this project can be resumed.

The objectives of this project are: 1) to select lines of mosquitoes susceptible or refractory to particular species of malaria; 2) to determine the mode of inheritance of susceptibility to a particular species of malaria; 3) to determine the susceptibility of selected mosquito lines to various geographic strains of the same malaria species and to other species of human and simian malaria; 4) to determine what physiologic factors may be involved in rendering mosquitoes refractory or susceptible to infection with human and simian malarias.

Lines of susceptible and refractory mosquitoes will be established from individual females and will be maintained by brother-sister crosses whenever possible. The mode of inheritance of susceptibility will be determined by standard methods of genetic analysis. The role of the mosquito neuro-endocrine system in regulating parasite development will be investigated using micro-surgical ablation and transplantation of various endocrine organs.

Development of genetically defined lines of mosquito vectors will provide important tools for a variety of research efforts. Susceptible and refractory lines will provide material for histo-chemical, physiological and biochemical investigations into the nature of the mosquito-malaria host parasite relationship.

Selected lines of natural malaria vectors refractory to 1 or more species of human malaria would be invaluable in biological control schemes involving vector displacement or genetic manipulation of vector populations.

These studies have been resumed and will be expanded to include a number of anopheline species and their relationships with a variety of human, simian, rodent and avian malarias.

Kew Words:

Vector genetics, Anopheles, malaria, mosquito physiology, mosquito endocrinology.

Publications:

- Omar, M.S., Gwadz, R.W., and Miller, L.H. Incorporation of nucleic acid precursors by Plasmodium cynomolgi in Anopheles balabacensis. Tropenmed. Parasit. In press.

Serial No. Z01 AI 00112-01 LPD
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Host controlled modifications in malaria parasites.

Previous Serial Number: None

Principal Investigator: Richard Carter

Other Investigators: Louis H. Miller

Cooperating Units: None

Man Years:

	NIAID LAB. STAFF	OTHER NIH PERSONNEL ASSIGNED TO PROJECT	GUEST WORKER	TOTAL
Total				
Professional				
Other				

Project Description:

The aim of this project is to study behavioral changes in malaria parasites and the role of the vertebrate host in controlling these changes. Particular attention is being given to the influence of the genetic constitution of the vertebrate host on the behavior of the parasite.

The model systems being used are P. berghei and P. yoelii in various inbred and outbred strains of mice. In preliminary work clones of P. berghei and P. yoelii have been prepared by dilution and are being adapted to appropriate strains of mice. In order to quantify behavioral characteristics of the parasites, techniques have been developed or adapted to enable the measurement of such characteristics as cycle length and red cell invasion preference. Other parameters relating to parasite growth rate will also be measured.

Key Words:

Rodent malaria, host-parasite modification, genetics.

Publications:

None

Project No. Z01 AI 00113-03 LPD
1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on dengue

Previous Serial Number: NIAID 67H

Principal Investigator: Dr. Leon Rosen

Other Investigators: Dr. Duane Gubler, Dr. Timothy Kuberski, Dr. Dwayne Reed, Dr. P.F.D. Van Peenen, Dr. Henri Kaeuffer, Dr. Archibald Guinea, Dr. William Bancroft

Cooperating Units: South Pacific Commission, Noumea, New Caledonia; U.S. Naval Medical Research Unit No. 2, Jakarta, Indonesia; U.S. Component, SEATO Laboratory, Bangkok, Thailand; Institut de Recherches Medicales, Papeete, Tahiti; Government of the Kingdom of Tonga; Government of Fiji; Government of the Cook Islands; Government of Nauru; U.S. Trust Territory of the Pacific Islands.

Man Years:	NIAID Staff	Guest Worker
Total:	16/12	1/12
Professional:	12/12	1/12
Other:	4/12	0/12

Project Description:

Objectives

1. To study the epidemiology of dengue and dengue hemorrhagic fever in the simple ecologic environment of Pacific islands.

2. To investigate the pathogenesis of severe and fatal dengue infection.

3. To improve laboratory methods for the detection of dengue viruses and their antibodies.

Methods Employed

The introduction of dengue type 1 infection to Pacific islands after an absence of approximately 30 years afforded an opportunity to observe the epidemiology of this dengue serotype under relatively simple ecologic conditions on Nauru, Ponape, and Fiji. Also, a flare-up of type 2 dengue in Tonga and Tahiti permitted further observations to be made on that serotype.

Major Findings

Epidemiology

In those instances where dengue type 1 outbreaks have occurred on islands recently infected by dengue type 2 (Nauru and Fiji) the type 1 disease appeared to be both more severe and more prevalent than did that previously caused by dengue type 2. The severity was manifested both by an apparently higher incidence of hemorrhagic manifestations as well as by an increased number of deaths. It is not known exactly why type 1 was more prevalent than type 2, but some possibilities are 1) greater inherent vector efficiency of the local Aedes aegypti, 2) higher average viremias and consequent higher vector efficiency for this reason, and 3) efficient transmission by vectors other than A. aegypti. This latter factor obviously played a role on the island of Ponape where A. aegypti was not found despite an intensive search. Aedes hakansonni, an endemic scutellaris species, was the most probable vector on that island.

Material was collected for viremia studies in Tonga, Nauru, and Fiji but work has been completed to date only on the material from Tonga - where it was found that the magnitude of dengue type 2 viremias was considerably lower for the smoldering outbreak which occurred there than that for the sharper type 2 outbreaks which occurred previously on Tahiti and in New Caledonia.

Pathogenesis

In the course of studies carried out in Tonga, Nauru, Jakarta, and Bangkok, unequivocal evidence was obtained both of severe hemorrhagic disease and of shock syndrome and death in patients older than one year of age experiencing primary dengue infections. These laboratory data confirm epidemiologic evidence previously obtained from a study of a dengue outbreak on the Island of Niue that severe and fatal dengue in individuals older than one year of age is not necessarily the result of sequential infection with dengue viruses.

The studies carried out during the recent Fiji outbreak should provide data on the question of whether secondary dengue infection with type 1 virus differs significantly from that of primary infection with the same virus. It is anticipated that approximately one-half the patients studied during the current outbreak had previously been infected with dengue type 2.

Collection of dengue virus strains for future pathogenesis studies was continued with additional strains obtained from patients in Vietnam, Indonesia, Singapore, Malaysia, Philippine Islands, and the New Hebrides.

Laboratory Technique

When virus isolation attempts were made on specimens from dengue hemorrhagic fever patients from Bangkok, it was found that the mosquito inoculation technique yielded two to three times as many isolates as did the standard plaque assay technique carried out by the SEATO Laboratory. Furthermore, by employing the fluorescent antibody technique to identify infected mosquitoes, and infected mosquitoes as antigen in complement-fixation tests, it was possible to identify isolates much faster than by previously employed techniques.

It had been noted previously that the use of the fluorescent antibody technique to identify mosquitoes infected with dengue was less satisfactory for types 3 and 4 than it was for types 1 and 2. It was found by further experimentation that the reason for this difference was that mosquitoes infected with the former types were less apt to show dengue antigen in their heads than were the latter. However, if the salivary glands of mosquitoes were examined, insects infected with all four dengue types were readily detected.

Significance to Biomedical Research and the Program of the Institute

Dengue viruses are increasing in significance as a cause of morbidity and mortality in Southeast Asia and Pacific islands - probably because of rapidly increasing urbanization and travel in those areas. Mosquito vectors of these viruses are ubiquitous in the same area, which includes many islands belonging to, or controlled by, the United States. Obviously, further understanding of the epidemiology of dengue and of the vectors of the disease would be of value.

Proposed Course of the Project

Further field studies on dengue outbreaks will be undertaken as appropriate opportunities occur. An attempt will also be made to write up for publication the extensive amount of data already on hand from outbreaks investigated previously.

Dr. Gubler, Guest Worker, will move to Jakarta, Indonesia, for two years beginning in July, 1975 to work on various aspects of dengue. He will work in close collaboration with this Section and most of the virus work will be done here.

Keyword Descriptors:

Dengue, dengue epidemiology, dengue hemorrhagic fever, dengue shock syndrome, *Aedes aegypti*, mosquitoes, Pacific islands, mosquito inoculation, Southeast Asia, vector efficiency, viremia

Publications:

Barnes, W.J.S. and Rosen, L.: Fatal hemorrhagic disease and shock associated with primary dengue infection on a Pacific Island. *Am. J. Trop. Med. Hyg.* 23:495-506, 1974.

Rosen, L. and Gubler, D.: The use of mosquitoes to detect and propagate dengue viruses. *Am. J. Trop. Med. Hyg.* 23:1153-1160, 1974.

Rosen, L.: Dengue type 3 infection in Panama. *Am. J. Trop. Med. Hyg.* 23:1205-1206, 1974.

Rosen, L.: Reoviruses. *In* Lennette, E. H., Spaulding, E.H. and Truant, J.P. (eds.): *Manual of Clinical Microbiology - Second Edition.* Washington, D.C., American Society for Microbiology, 1974, pp 735-739.

Rosen, L.: Reoviruses. *In* Hubbert, W.T., McCulloch, W.F. and Schnurrenberger, P.R. (eds.): *Diseases Transmitted from Animals to Man, Sixth Edition*. Springfield, Ill., Charles C. Thomas, 1975, pp. 919-921.

Yii, C-Y., Chen, C-Y., Chen, E-R., Hsieh, H-C., Shih, C-C., Cross, J.H., and Rosen, L.: Epidemiologic studies of eosinophilic meningitis in southern Taiwan. *Am. J. Trop. Mec. Hyg.* 24:447-454, 1975.

Rosen, L.: Angiostrongyliasis. *In* Hubbert, W.T., McCulloch, W.F., and Schnurrenberger, P.R. (eds.): *Diseases Transmitted from Animals to Man,* Sixth Edition. Springfield, Ill., Charles C. Thomas, 1975, pp. 567-571.

Project No. Z01 AI 00114-05 LPD
1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on arboviruses other than dengue

Previous Serial Number: NIAID 67C

Principal Investigator: Dr. Robert B. Tesh

Other Investigators: Dr. Leon Rosen, Dr. Duane J. Gubler

Cooperating Units: Department of Tropical Medicine and Medical Microbiology, University of Hawaii School of Medicine, Honolulu; Yale Arbovirus Research Unit, Yale University School of Medicine, New Haven, Conn.; U.S. Naval Medical Research Unit No. 2, Taipei, Taiwan; University of California Center for Medical Research, University of Malaya, Kuala Lumpur, Malaysia; Laboratory of Central Nervous System Studies, NINDS, NIH; U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland; School of Public Health, University of Teheran, Iran.

Man Years:

Total:	14/12
Professional:	12/12
Other:	2/12

Project Description:

Objectives

1. To study the epidemiology of arthropod-borne virus infections other than dengue of public health importance on the Pacific islands and in Southeast Asia.

2. To study the genetic basis of susceptibility and resistance to chikungunya virus infection in selected geographic strains of *Aedes albopictus*.

3. To elucidate the epidemiology of sandfly fever through serologic surveys of human populations living in endemic areas of the disease and by laboratory studies of viruses comprising the Phlebotomus fever group.

4. To examine the possibility of transovarial transmission of selected arboviruses by experimentally infected mosquitoes.

Methods Employed

Studies of experimental arbovirus infection in mosquitoes after intrathoracic inoculation or feeding were done utilizing laboratory colonies of *Aedes albopictus* and *Culex fatigans*. Culture and titration of insect specimens were done in tube or microplate cultures of Vero, RK_{13} or $LLC\text{-}MK_2$ cells. Serologic studies of human sera were done by standard complement fixation, hemagglutination-inhibition, or plaque neutralization tests.

Major Findings

A. Variation among geographic strains of *Aedes albopictus* in suscepti- to chikungunya virus infection.

Using chikungunya as a model of arbovirus infection, the susceptibility of 16 different geographic strains of *Aedes albopictus* to oral infection was studied. Two types of variation were observed. One was differential susceptibility to infection with the virus. Chikungunya susceptibility among different geographic strains varied from 32 to 97%. The second difference was variation in the quantity of virus present in infected mosquitoes after a standard incubation period. Mean virus titers of infected mosquitoes of different geographic strains varied almost 1000-fold. These observed variations in both chikungunya susceptibility and virus content among *A. albopictus* strains suggest that differences also occur in their vector competence.

Attempts to develop increasingly resistant or susceptible mosquito lines through genetic selection were unsuccessful. Infection rates did not change significantly despite 3 to 6 generations of selective inbreeding. In contrast, crosses between strains of high and low chikungunya susceptibility yielded hybrid mosquitoes with infection rates and mean virus titers intermediate between those of the parent colonies. These data suggest that at least one factor controlling the susceptibility of *A. albopictus* to chikungunya infection is genetic. Interestingly, two lines of *A. albopictus* with a marked difference in susceptiblity to oral infection with dengue viruses were equally susceptible to oral infection with chikungunya virus, suggesting that the mechanism(s) controlling susceptibility to these two arboviruses in *A. albopictus* are distinct.

B. Serologic studies of arbovirus infection among human populations of the South Pacific

Plaque reduction neutralization tests, using 5 group A arboviruses (chikungunya, Ross River, Getah, Bebaru and Sindbis), were done on several thousand sera from human populations in 48 Southeast Asia and Pacific island localities. The plaque neutralization method appears to be more specific for identifying group A arbovirus infections than the hemagglutination-inhibition test which has been used extensively in the past in serologic surveys. Specificity of the plaque neutralization test was first determined by examining convalescent sera from patients with known alphavirus infections. Chikungunya-specific neutralizing antibodies were demonstrated in sera of persons living in South Vietnam, Burma, Malaysia, Indonesia (Kalimantan and Sulawesi), as well as Luzon, Marinduque, Cebu and Mindanao Islands in the Philippines. Evidence of considerable Ross River virus infection was found among populations living in West New Guinea, Papua New Guinea, the Bismarck Archipelago, and the Solomon Islands. Ross River virus is the presumed etiologic agent of epidemic polyarthritis and has not been demonstrated previously outside of Australia. There appeared to be no geographic overlap in the occurrence of chikungunya and Ross River viruses, with separation in their distribution corresponding with Weber's line in the Pacific. Antibody rates to Sindbis and Getah viruses were low and no evidence of human infection with Bebaru virus was found. A manuscript was prepared describing the prevalence and geographic distribution of Group A arbovirus antibodies among human populations in Southeast Asia and the Pacific islands.

C. Laboratory studies of transovarial transmission of La Crosse and other arboviruses by mosquitoes

Recent reports of transovarial transmission of La Crosse, Keystone and snowshoe hare viruses of the California encephalitis serogroup, family Bunyaviridae, by naturally infected *Aedes* mosquitoes, prompted us to test whether vertical transmission of La Crosse and several related arboviruses might occur in additional mosquito species. Transovarial transmission of La Crosse virus by experimentally infected *Aedes albopictus* females to 2.7% of their F_1 generation offspring was demonstrated. Progeny of both sexes were infected. Mean virus titers in parent mosquitoes and infected F_1 generation adults were $10^{4.6}$ and $10^{3.4}$ PFU/insect, respectively. The La Crosse infected offspring were randomly distributed among the female parents. After 2 serial passages in *A. albopictus*, a marked change occurred in the plaque morphology of the virus but this had no apparent effect on the subsequent vertical transmission rate. In contrast, transovarial transmission did not occur in La Crosse infected *Culex fatigans* or in *A. albopictus* and *C. fatigans* infected with VSV-Indiana (a rhabdovirus), Cache Valley, Batai, Arumowot, Itaporanga (bunyaviruses), chikungunya or Ross River (alphaviruses) viruses. Results of this experiment indicate that the La Crosse model might be useful in studying the mechanism of transovarial transmission in additional mosquito species. Our data also suggest that vertical transmission in mosquitoes is a phenomenon unique to the California encephalitis group and is not a mechanism common to all bunyaviruses.

D. Failure of rubella virus to replicate in mosquitoes

Rubella virus recently has been included in the togavirus family because of physicochemical and morphologic relationships to the alpha- and flaviviruses. Although rubella virus is antigenically distinct from arboviruses of the A and B serogroups, several workers have suggested that it might be an arbovirus. In order to obtain more information on this point, the survival of rubella virus was studied following parenteral inoculation into mosquitoes. This method has been used extensively to identify new arboviruses and to determine their potential arthropod host range.

Rubella virus (M-33 strain) was inoculated intrathoracically into female A. albopictus and C. fatigans which were subsequently maintained at 32°C and tested at weekly intervals. Rubella virus was present in mosquitoes tested immediately after inoculation but was not detected in insects sampled on days 7, 14 and 21 post-inoculation. These data indicate that rubella virus did not replicate in the mosquitoes tested and offer further evidence that it is not an arbovirus.

E. Serologic response of volunteers infected with sandfly fever virus

In the past the diagnosis of sandfly fever has been made solely on clinical and epidemiologic evidence, due to the lack of a specific serologic test to diagnose infection. Recently, clinical data and serial serum specimens from 26 volunteers infected with the Sicilian strain of sandfly fever virus were obtained from the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick. All sera were tested by standard complement fixation (CF), hemagglutination-inhibition (HI) and plaque reduction neutralization (PRN) methods. Little CF antibody was detected in convalescent sera but most subjects developed significant rises in HI antibodies. All infected subjects developed specific neutralizing antibodies with titers ranging from 1:40 to 1:2560. Of the three serologic tests performed, the PRN method appeared to be the most sensitive for detecting antibodies to Phlebotomus fever viruses. The development of this test now offers a method to diagnose sandfly fever virus infection and it also provides a useful tool for performing epidemiologic serum surveys in endemic areas of the disease.

F. Attempted virus isolation from Asian sandflies

The Phlebotomus fever arbovirus serogroup currently consists of 22 distinct virus types isolated in the Mediterranean region, Africa, Central Asia and tropical America. Although there is some geographic overlap, each region appears to have a different serotype or combination of serotypes. No Phlebotomus fever group viruses have yet been obtained from eastern Asia, although sandfly fever has been reported from Burma and China. In an attempt to obtain an isolate from this region, 15,635 Malaysian sandflies (males and females) of the genera Phlebotomus and Sergentomyia were processed for virus isolation. These specimens were collected by workers at the University of California Center for Medical Research in Kuala Lumpur. Unfortunately no viruses were obtained from 138 insect pools inoculated into tube cultures of Vero cells. In view of the negative results, this study has been discontinued.

G. Serologic studies on the epidemiology of sandfly fever

Following the development of the plaque reduction neutralization test to detect antibodies against Phlebotomus fever group viruses, it was decided to examine sera from persons living in endemic areas of sandfly fever in order to learn more about the epidemiology of the disease. During the past year, a large number of human sera from Greece, Egypt, Sudan, Nigeria and Iran have been obtained and tested against 6 Phlebotomus fever group viruses (Naples, Sicilian, Arumowot, Karimabad, Salehabad and SudAn 754-61) known to occur in the Old World. Although still in progress, these studies indicate a high prevalence of infection with one or more Phlebotomus fever serotypes among many residents of the Eastern Mediterranean. Among indigenous populations, infection appears to occur frequently in childhood, since many persons have antibodies by the age of 20 years. To date, serologic evidence of human infection has been formed with 4 (Naples, Sicilian, Karimabad and SudAn 754-61) of the 6 virus serotypes tested. Of these, the Naples virus type appears to be the most prevalent. Additional serologic surveys in other regions are planned.

Significance to Biomedical Research and the Program of the Institute

Chikungunya, a group A arbovirus, has been responsible for major epidemics of dengue-like illness among human populations in Africa, India, the Philippines, and several countries of Southeast Asia. Since dengue and chikungunya infection are similar clinically and as both agents may occur simultaneously in the same area, differentiation of illness produced by the two viruses is of obvious public health importance. Like dengue, Aedes aegypti has been implicated as the principal urban vector during epidemics of chikungunya disease. Despite a common vector, overlapping geographic distribution, and clinical similarity with dengue, however, isolations of chikungunya virus from other mosquito genera (Anopheles, Culex and Mansonia), its sporadic appearance, as well as its occurrence in dengue-free areas of Africa suggest that the ecology of the two viruses is different. These studies are designed to learn more about the epidemiology of chikungunya disease.

Ross River, a second Group A arbovirus, is the presumed etiologic agent of epidemic polyarthritis, a disease of some public health importance in Australia. Results of our serologic surveys indicate that the virus also occurs in New Guinea, the Bismarck Archipelago and several of the Solomon islands, extending its known geographic distribution. Studies of epidemic polyarthritis patients in Australia suggest that the disease may represent a hypersensitivity reaction following Ross River virus infection, since persons manifesting rash and arthritic symptoms already have antibodies to the agent and attempts at virus isolation have been unsuccessful. In this respect, epidemic polyarthritis is analogous to the arthritis associated with rubella infection.

Studies of the genetic basis of mosquito susceptibility and resistance to chikungunya virus infection were initiated because of current interest in the possibility of using genetic techniques for the control of insect pests. Theoretically, in the case of an arthropod-borne disease, it might be possible to interrupt disease transmission by replacement of vector (susceptible) forms of a species by non-vector (refractory) varieties. Support for this hypothesis has been provided by laboratory studies of mosquitoes infected with several different malarial and filarial parasites, which have demonstrated genetic factors controlling susceptibility as well as the feasibility of developing increasingly resistant strains by genetic selection. Such studies have not been reported previously with mosquito-borne viruses.

Results of our studies with A. albopictus and the chikungunya virus model indicated marked differences in susceptibility as well as virus content among various geographic mosquito strains. These findings suggest that differences also occur in their vector competence. While these phenomena were demonstrated in laboratory colonies, presumedly similar variations occur among natural mosquito populations. If this case applies to other arboviruses and their insect vectors, it may be one explanation for the observed differences in the character and occurrence of mosquito-borne virus epidemics in various geographic regions. These data also have important implications for the potential biologic control of mosquito vectors of human disease.

La Crosse virus, a member of the California encephalitis arbovirus group, has been the agent most frequently associated with human cases of California encaphilitis in the United States. Recent evidence of transovarial transmission of this virus by Aedes triseriatus in Wisconsin and Ohio demonstrates an efficient mechanism for overwintering of the agent and maintenance in the mosquito population. This is the first evidence of transovarial transmission of an arbovirus by mosquitoes. Recent reports of isolations of two additional California encephalitis virus serotypes from naturally infected mosquito larvae indicate that this mechanism may be a property shared by other viruses of the California serogroup. The demonstration of La Crosse virus vertical transmission by A. albopictus (an unnatural host for the virus) suggests that the La Crosse model might be studied in other mosquito species to elucidate the mechanism of transovarial virus transmission.

Sandfly fever has long been recognized as a disease of considerable public health and military importance in the Middle East and Central Asia. Previous work on sandfly fever has been limited mainly to clinical observations of patients and to studies of the mode of transmission because of difficulties in culturing the etiologic agents and the lack of a specific serologic test for determining immunity. Now, however, with the availability of tissue culture for primary virus isolation and for use in plaque neutralization tests, the necessary tools are available for a thorough epidemiologic study of sandfly fever. A number of basic facts about the epidemiology of the disease are still unknown, such as the true prevalence of infection among indigenous populations, the clinical spectrum of sandfly fever, the duration of immunity, the prevalence of different virus serotypes, the sandfly species involved in virus transmission, the maintenance mechanism of the viruses from season to season, and the possibility of animal reservoirs. The serologic studies now in progress on human populations living in endemic areas of the disease are designed to answer some of these questions.

Proposed Course of the Project.

Additional laboratory studies with chikungunya virus and various geographic strains of A. albopictus are planned. One important question, yet to be answered, is the effect of laboratory colonization of mosquitoes on their subsequent virus susceptibility. Do inbred laboratory colonies differ significantly from wild populations in their virus susceptibility? An answer to this question is essential before any serious attempt at biologic replacement of wild mosquito populations can be made. We also plan to investigate the possibility of genetic differences in mosquito infectivity among chikungunya virus strains from various geographic regions.

Serologic surveys of the prevalence of human infection and the geographic distribution of Phlebotomus fever group viruses will be continued. Plans are also being made to begin field studies on the epidemiology of sandfly fever in Egypt and/or Iran. These studies will be in collaboration with workers at the NAMRU-3 unit in Cairo and the School of Public Health in Teheran.

Keyword Descriptors

Chikungunya virus, mosquito susceptibility to arboviruses, prevalence of alphaviruses, La Crosse virus, transovarial arbovirus transmission, Phlebotomus fever group viruses, serologic response in sandfly fever, plaque reduction neutralization test, *Aedes albopictus*.

Publications

Tesh, R.B. and Johnson, K.M.: Vesicular stomatitis. In Hubbert, W.T., McCulloch, W.F. and Schnurrenberger, R.P. (eds): *Diseases Transmitted from Animals to Man*. Sixth Edition. Charles C. Thomas, Springfield, Illinois, pp. 897-910, 1975.

Noble, G.A. and Tesh, R.B.: *Monoecocestus diplomys* sp. n. (Cestoda: Anoplocephalidae) from the rat *Diplomys darlingi*. *J. Parasitol*. 60:605-607, 1974.

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Tesh, R.B.: Multiplication of Phlebotomus fever group arboviruses in mosquitoes after intrathoracic inoculation. *J. Med. Entomol*. 1975 (in press).

Tesh, R.B. and Gubler, D.J.: Laboratory studies of transovarial transmission of La Crosse and other arboviruses by *Aedes albopictus* and *Culex fatigans*. *Am. J. Trop. Med. Hyg*. 1975 (in press).

Tesh, R.B. and Chaniotis, B.N.: Transovarial transmission of viruses by phlebotomine sandflies. *Ann. N. Y. Acad. Sci*. 1975 (in press).

Project No. Z01 AI 00115-01 LPD
1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the natural history of influenza virus in swine

Previous Serial Number: None

Principal Investigator: Dr. Gordon D. Wallace

Other Investigators: Dr. John Shanley

Cooperating Units: Animal Science Department, College of Tropical Agriculture, University of Hawaii and Division of Animal Industry, Hawaii State Department of Agriculture, Honolulu, Hawaii

Man Years:

Total	25/12
Professional:	23/12
Other:	2/12

Project Description:

Objectives

To determine the method(s) by which influenza virus is maintained in swine herds.

Methods Employed

Observations on the epidemiology of influenza in swine indicate that swine influenza virus is maintained in swine herds by methods other than periodic re-introduction from the outside. Dr. Richard Shope developed the classic hypothesis, supported by a large series of experiments, that swine lungworm larvae while living in their earthworm host serve as an extra mammalian reservoir of virus. Other data, however, from experiments and epidemiologic observations conducted in Czechoslovakia, supported by independent epidemiologic observations in Wisconsin, USA, suggest that some swine may become chronically infected in the absence of lungworms and periodically shed virus over a long period of time. It was shown experimentally that pigs born to sows infected during pregnancy, or born to chronically infected sows in which infection is activated during pregnancy, develop immunologic tolerance and harbor demonstrable virus themselves for long periods.

These hypotheses (lungworm transmission, and chronic shedding of virus), were developed primarily from experimental data and it is intended to test them in Hawaii and possibly on other Pacific islands by careful long-term prospective seroepidemiologic and virologic study of naturally infected swine herds. This can be accomplished to advantage on Pacific islands because herds are relatively isolated, and herds with influenza but not infected with lungworms probably can be found.

In addition to standard serologic and virus-isolation techniques, organ culture is being employed. This method should increase the probability of isolating virus from chronically infected animals. Demonstration of transplacental transmission and chronic infection with influenza virus in swine will be attempted experimentally with strains of virus isolated from naturally infected swine.

Major Findings

Sera, mostly obtained at slaughter, from 800 adult swine representing 65 herds on the island of Oahu were tested for hemagglutination-inhibition (HI) antibody to A/swine/Wisconsin/67 (HSw1N1) and A/Hong Kong/8/68 (H_3N_2) strains of virus. Antibodies were detected in swine from 45% of the herds. About 20% of the sera tested had antibody (≥1:20) only to A/swine, 8% only to A/HK and 5% to both strains. Titers to A/HK generally were low (1:20 - 1:40), but some sera had titers equal to or greater than 1:80. Sera positive to A/HK were tested against two other more current human strains (A/England/42/72 and A/Port Chalmers/1/73), however, with few exceptions, titers were the same. Some of these sera are currently being tested for antibody to N_1 and N_2 antigens by the neuraminadase inhibition test.

After additional seroepidemiologic investigations, 5 herds were selected for detailed study, based on the following criteria:
1) results of HI tests on swine of various ages suggested endemicity of virus, 2) relative geographic isolation with infrequent or no introduction of swine from other farms, 3) disease in new-born pigs compatible with influenza (stillbirths, weak pigs, runt pigs - epidemic influenza in adult animals has not been observed on Oahu).
4) cooperative attitude of farm owners. To date, virus isolation has been attempted, unsuccessfully, from 6 weak or runted suckling pigs, 2 each from 3 farms. Pooled feces from about 20 pens holding 10 to 20 adult breeding animals each on one of the 5 farms have been examined for lungworm ova, but none were observed.

Significance to Biomedical Research and the Program of the Institute

New information on the natural history of swine influenza will be of considerable biologic interest and may provide insight into the natural history of human influenza.

Proposed Course of the Project

The selected swine herds will be studied prospectively as outlined above. Virus strains isolated will be used in experiments designed to evaluate vertical transmission and the development of chronic infection. In addition, we have the opportunity to determine if human A/HK strains have become established in swine in Hawaii. Currently, swine sera are being screened against both A/HK/68 and A/Port Chalmers. (B/Hong Kong antigen will also be included, because little information is available on whether type B influenza virus is naturally transmitted from man to swine.) Since there is serologic evidence suggesting simultaneous circulation of A/swine and A/HK strains in the same herds, there is the possibility of a recombinant strain developing. The discovery of such a virus would be of considerable interest. Consideration is also being given to initiating a prospective study on vertical transmission of influenza virus in humans. There is very little definitive information on this subject. However, such a study would have to be done during an epidemic.

Keyword Descriptors:

Influenza virus, chronic infection, natural history, organ culture, swine lungworm, transplacental transmission.

Publications:

None

Project No. Z01 AI 00116-01 LPD
1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Investigations on the etiology of non-gonococcal urethritis (NGU)/Counterimmunoelectrophoresis in the detection of dengue virus and *Angiostrongylus cantonensis* antigens.

Previous Serial Number: None

Principal Investigator: Dr. Timothy T. Kuberski

Other Investigators: Dr. Robert Shaw, Dr. Leon Rosen and Dr. Gordon D. Wallace

Cooperating Units: U.S. Navy Environmental & Preventive Medicine Unit No. 6, Pearl Harbor Naval Shipyard, Honolulu, Hawaii and Dr. Sompone Punyagupta, Chief, Division of Gastroenterology and Tropical Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

Man Years:

Total:	12/12
Professional:	10/12
Other:	2/12

Project Description:

Objectives

VD Study

This study is designed to determine the characteristics of non-gonococcal urethritis (NGU) in a defined susceptible population. The broad objectives of this study will be to: 1) Characterize the epidemiology of the disease in the population; 2) Describe the clinical aspects of NGU; 3) Perform laboratory investigations for the more likely etiologic agents suspected to be involved in the disease.

Counterimmunoelectrophoresis (CIEP) study

1) To study the use of counterimmunoelectrophoresis (CIEP) as a method in the routine detection of dengue virus infected mosquitoes; 2) To determine if the counterimmunoelectrophoresis (CIEP) technique is capable of detecting antigen(s) of *Angiostrongylus cantonensis* in the cerebrospinal fluid (CSF) of its natural host, the rat. If these antigens can be demonstrated in the CSF of the rat, this procedure might be refined enough for use in the diagnosis of human meningitis due to *A. cantonensis*.

Methods Employed

VD study

Any male patient entering the Navy infirmary complaining of urethritis (pain, burning, itching of distal urethra, or urethral discharge) will be evaluated provided he has not taken any antibiotics in the past week. The estimated sample size of the study group will be 300 patients plus 300 controls (men entering without genitourinary complaints). If a discharge is present two slides will be prepared of the exudate. One slide will be stained for gonococci; if no evidence of gonococci exists the patient will presumptively be considered as having NGU. The second unstained slide will be examined for trichomonas.

The patients and controls will complete a portion of a questionnaire relating to epidemiology and clinical manifestations. The questionnaire will be verified and completed by medical personnel. An acute blood specimen will then be obtained.

A physical examination will be recorded by a physician and three urethral swabs taken. The swabs will be processed immediately into the following: 1) Transport media for chlamydia and viral studies; 2) (a) Thayer-Martin media to rule out the presence of *Neisseria gonorrhea*, (b) blood agar media to define the urethral flora, (c) mycoplasma media; 3) Trichosel broth for trichomonas.

The patient will then be asked to urinate into a sterile container. The urine sediment will be examined for cytology as well as cultured for chlamydia and trichomonas.

A standard regimen of tetracycline will be supplied to the patient and he will be asked to return in two weeks for a follow-up evaluation and a convalescent blood specimen.

Counterimmunoelectrophoresis (CIEP) study

1. Dengue infected mosquitoes. Mouse ascitic fluid prepared against the four prototype dengue viruses was used as standard antisera. Aedes albopictus, experimentally infected by the microinoculation technique with prototype dengue viruses, were sonicated, centrifuged and the supernatant used as antigen.

2. A. cantonensis infection. Homogenized male and female adult A. cantonensis were used as antigen for producing antisera. Hyperimmune sera against soluble A. cantonensis antigen was prepared in rabbits. Experimental A. cantonensis infection consisted of infecting twenty adult laboratory rats by intragastric instillation of approximately 100 third stage larvae. A serum specimen was obtained at the time of exposure as an acute specimen. Groups of 5 rats were exsanguinated and CSF removed at 10 day intervals for approximately 40 days. An additional group of five uninfected rats had CSF and serum removed for control purposes. A commercially available CIEP system, Hyland Austigen II, was used.

Major Findings

VD study

The study is now only several weeks old and as yet no findings can be reported. Most of the time spent on this project to date was in the development of techniques and standardization of methods.

Counterimmunoelectrophoresis (CIEP) study

1. Dengue infected mosquitoes. The CIEP technique did not appear sensitive enough, using the anti-dengue mouse ascitic fluid, to detect the presence of homologous virus in a single mosquito. A number of infected mosquitoes were necessary for a precipitin band to form (frequently more than five) and the number of infected mosquitoes necessary to give a precipitin band with homologous antisera was inconsistent. In addition, there were differences in the sensitivity of the various antisera in detecting infected mosquitoes, some antisera forming precipitin bands more readily with less antigen. Use of other antisera (human and monkey) resulted in the formation of a precipitin reaction in control uninfected mosquitoes which could not be completely absorbed out.

2. A. cantonensis infection. The serum and CSF from both control and experimentally infected rats were tested for the presence of either A. cantonensis antibody or antigen using the CIEP technique. All of the CSF specimens were negative for the presence of detectable antibody or antigen. However, circulating antigen was detected in the serum of all rats on the 30th day after infection. This correlates with the movement of the worms from the central nervous system to the pulmonary vasculature.

Cerebrospinal fluid from human patients thought to have eosinophilic meningitis were also examined using the CIEP technique. No A. cantonensis antigen or antibody could be detected in these specimens.

Significance to Biomedical Research and the Program of the Institute

VD study

The increase of veneral diseases, particularly gonorrhea, is well established and understandably is one of today's major public health problems. An increasingly apparent problem however, is the sizable proportion of cases of acute urethritis, often called gonorrhea and treated as such, which may not be due to the gonococcus. This entity has been classified as non-gonococcal urethritis (NGU) or non-specific urethritis (NSU). Comparatively little is known about NGU in the United States primarily because it has not been investigated systematically and is not included in the venereal disease statistics of the Public Health Service; therefore its incidence, epidemiology and clinical manifestations are not well delineated. A number of possible etiologies for this condition have been evaluated, but a proven, primary etiology for the majority of NGU cases has not been established. The major categories of agents or mechanisms implicated in causing this syndrome have been chlamydia (TRIC agents), T-strain mycoplasmas, trichomonads, bacterial urethral flora and hypersensitivity phenomena. Reviewing the literature on each of these possible agents indicates that some or all, may account for a small percentage of NGU cases, but as yet none has been convincingly established as a major causative agent.

An attempt will be made to define some of the factors in the epidemiology of NGU and more importantly, to correlate these with the clinical and laboratory findings. A controlled study is necessary to gain some insight into the pathogenesis of NGU because to date few consistent observations have been synthesized, possibly because of the heterogeneous etiology which may be involved.

Counterimmunoelectrophoresis (CIEP) study

A recent refinement in gel diffusion has been the counterimmunoelectrophoresis (CIEP) technique. The use of CIEP in the diagnosis of viral diseases (other than hepatitis B) has not been extensively explored. Gel diffusion techniques have been used with the dengue viruses and immunodiffusion has been reported to be useful in typing and in the antigenic analysis of the dengue viruses. It was felt the CIEP technique might offer a technically less difficult and time consuming alternative to the currently used IFA method of identifying dengue infected mosquitoes and in addition, prove to be a more useful technique for remote laboratories or field work.

CIEP has also been used in the diagnosis of meningitis and has been shown to be capable of rapidly and specifically identifying antigens in the cerebrospinal fluid (CSF) of patients with meningitis due to the major bacterial causes of this disease. A reasonable extension of these observations might be its applicability to *A. cantonensis* infections of the central nervous system. Currently there is no reliable serologic method for the diagnosis of eosinophilic meningitis due to *A. cantonensis*.

Proposed Course of the Project

VD study

The study will continue to completion of 300 NGU study patients and 300 controls. There will be a review of accumulated data periodically throughout the study to see if any modifications in methods or protocol are necessary to improve the study. From the results of this study more comprehensive investigations into particular facets of NGU can be pursued.

Counterimmunoelectrophoresis (CIEP) study

The sensitivity of the CIEP technique is in great part dependent on the potency of the antisera used. For detecting dengue infected mosquitoes developing a more potent antisera, not cross-reacting with uninfected mosquitoes, might improve the capabilities of this technique. Since the anti-dengue mouse ascitic fluid is the most potent antisera available which does not cross react with normal mosquito antigen, this study has been terminated. The use of CIEP in the detection of *Angiostrongylus cantonensis* antigen in the CSF of human infections has also been phased out because the preliminary experiments have proven unsuccessful.

Keyword Descriptors:

Venereal disease, non-gonococcal urethritis, chlamydia, mycoplasma, Trichomonas, *Angiostrongylus cantonensis*, dengue, counterimmunoelectrophoresis.

Publications:

None

Serial No. Z01 AI 00117-05 LPD
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Pathogenesis of the malarial paroxysm

Previous Serial Number: NIAID 134

Principal Investigator: Franklin A. Neva

Other Investigators: Michael Frank, M.D.; John Atkinson, M.D.; and Louis H. Miller, M.D.

Cooperating Units: Laboratory of Clinical Investigation, Section on Medical Parasitology, NIAID

Project Description:

This project has been cancelled.

Serial No. Z01 AI 00118-07 LPD
1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the natural history of Toxoplasma gondii, the mechanism of its transmission to man, and its public health importance in the Pacific area.

Previous Serial Number: 67D

Principal Investigator: Dr. Gordon D. Wallace

Other Investigators: Dr. Alistair Philip, Department of Pediatrics. University of Hawaii School of Medicine, Honolulu, Hawaii

Project Description:

This project has been terminated.

Project No.Z01 AI 00119-07 LPD
1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Susceptibility and Resistance of Mosquitoes to Dengue

Previous Serial Number: NIAID 67 I

Principal Investigator: Dr. Duane J. Gubler

Other Investigators: Dr. Leon Rosen

Cooperating Units: Department of Tropical Medicine and Medical Microbiology, University of Hawaii School of Medicine, Honolulu

Man Years:	NIAID staff	Guest worker
Total:	4/12	11/12
Professional:	2/12	11/12
Other:	2/12	0/12

Project Description:

Objectives

1. To select strains of Aedes albopictus which are either highly resistant or highly susceptible to infection with dengue viruses.

2. To study the genetic basis of susceptibility to infection with dengue viruses.

3. To study the relationship between salivary gland infection and transmission of dengue viruses.

Methods Employed

Strains of A. albopictus from different geographic regions were screened for susceptibility to infection with dengue viruses. Attempts were made to increase or decrease susceptibility in certain mosquito strains by selective inbreeding. Reciprocal and backcrossing experiments were made to determine the mode of inheritance controlling vectorial capacity.

The mechanism of salivary gland infection and the relationship between salivary gland infection and transmission were studied by the direct fluorescent antibody technique and by transmission studies using an artificial feeding technique.

Major Findings

The comparative susceptibility to oral dengue virus infection of 13 geographic strains of Aedes (Stegomyia) albopictus was studied. Significant variation in susceptibility for all 4 dengue serotypes was observed among the geographic strains tested. Mosquitoes which were more susceptible to infection with one dengue serotype were also more susceptible to the other dengue serotypes and vice versa, suggesting that the factor or factors controlling susceptibility are the same for all 4 serotypes.

Significant quantitative differences in the oral threshold level of infection (ID_{50}) to dengue 2 virus were also observed among these same mosquito strains. There was a direct relationship between the amount of virus ingested and the infection rate in a given mosquito strain. The oral ID_{50} of the most susceptible and least susceptible strains varied by 100-fold.

Crossing experiments between susceptible and resistant mosquito strains produced hybrid progeny with intermediate susceptibility suggesting genetic control. In most of the mosquito strains tested, however, susceptibility was very stable and could not be increased or decreased by selective inbreeding. One exception was the strain from Poona, India. Susceptibility to oral infection with dengue 2 virus was decreased by selective inbreeding from 74% to 12% in 2 generations. Further selection, however, failed to produce a completely resistant line. The actual mode of inheritance of susceptibility to dengue viruses therefore, could not be determined.

Experiments on the comparative susceptibility of the Poona resistant line and the parent Poona colony strain to infection with dengue 2 by intrathoracic inoculation revealed no difference between the 2 groups of mosquitoes. These data indicate that the actual "barrier" to infection with dengue viruses is in the midgut.

The relationship between susceptibility of the mosquito and vector competence is not yet clear. Most of these studies have dealt only with infection without regard to whether or not transmission will occur. Virus growth in the various organs was traced in the Oahu A. albopictus and there was good correlation between infection in the whole mosquito and infection in the salivary glands. To study the relationship between salivary gland infection and transmission, however, a new technique was developed which utilized feeding mosquitoes on a suspension consisting of equal parts of washed human erythrocytes, calf serum and 10% sucrose. Transmission rates of A. albopictus were related to the amount of salivary gland tissue infected with dengue virus. The average infective mosquito transferred 10^3 MID_{50} while feeding, but it was not uncommon for mosquitoes to transmit over 10^4 MID_{50}. The largest quantity of virus transferred in these experiments was $10^{5.7} MID_{50}$. Transmission of virus occurred even though the insect only probed without taking blood. The earliest transmission after oral infection was 10 days. The data show that transmission occurs in most mosquitoes after the salivary gland infection has reached a certain level. The implication for this mosquito strain is that once the insect is infected, virus replication in the salivary glands, and eventually transmission, will occur if the mosquito lives long enough.

Significance to Biomedical Research and the Program of the Institute

Dengue viruses are widespread in Asia and the Pacific and outbreaks of the disease associated with severe hemorrhage or shock make dengue one of the most important public health problems of that area. Control of the vector is presently the only available means of controlling the disease. The results of the present study suggest that it might be possible to achieve disease control in an area by replacing a susceptible vector population with a more refractory strain of the same species. Even though crossing with the indigenous strain would occur, the data indicate that the resulting hybrids would be more resistant to infection with all 4 dengue serotypes. It is possible therefore, that even though low level transmission of dengue was maintained, the vector competence of the hybrid population would be too low to support large outbreaks of dengue fever.

Proposed Course of the Project

The laboratory aspects of this project have been terminated. The Principal Investigator plans to move to Indonesia in July to initiate a collaborative project on dengue vector competence involving the Pacific Research Section, NIAID, Honolulu, Hawaii; NAMRU-2 in Jakarta and the Indonesian Directorate of Communicable Disease Control, Jakarta, Indonesia.

Keywords

Mosquitoes, dengue, vector competence, virus susceptibility, virus transmission.

Publications:

Gubler, D.J. and Bhattacharya, N.C.: A quantitative approach to the study of bancroftian filariasis. Amer. J. Trop. Med. Hyg. 23:1027-1036, 1974.

Project No. Z01 AI 00120-07 LPD
1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Observations on two recently discovered coccidian parasites with similarities to Toxoplasma gondii

Previous Serial Number: NIAID 67 J

Principal Investigator: Dr. Gordon D. Wallace

Other Investigators: Dr. J. K. Frenkel

Cooperating Units: School of Medicine, University of Kansas, Kansas City

Man Years:

Total	1/12
Professional:	1/12
Other:	0/12

Project Description:

Objectives

To elucidate the life cycles and biologic characteristics of the newly discovered parasites, particularly their relationships to Toxoplasma and their potential to infect man.

Methods Employed

The parasites were isolated during studies on the natural history of T. gondii when oocysts resembling T. gondii, found in feces of stray cats in Hawaii, were administered orally to laboratory mice.

Major Findings

One of the parasites, previously referred to as WC1170, has been described in detail in last year's report. Dr. J. K. Frenkel has proposed the name of Hammondia hammondi for this parasite. Morphologically its stages are remarkably similar to those of T. gondii, but major differences in the life cycles were noted. Its host range appears to be limited and it is relatively non-pathogenic for laboratory animals. Furthermore, there is no evidence that it is of medical importance.

During studies on *H. hammondi*, cysts of another parasite were recognized in tissues of mice treated with cortisone acetate and fed fecal material from a stray cat. The cysts were typical of *Besnoitia*, a sporozoan parasite with an unknown life cycle that is of veterinary medical importance. Feeding mice infected with the *Besnoitia* cyst to cats led to fecal shedding of isospora-type oocysts measuring 13x16µ and a cat-mouse-cat cycle was established in the laboratory. Similar oocysts were found in the feces of the stray cat referred to above. Thus a *Besnoitia* species similar to *Besnoitia* in cattle, horses, reindeer, impala, other mammals and reptiles, has been shown to be a coccidian of cats capable of being spread by fecal contamination. (*Besnoitia* has not been reported in man.) This is the fourth mammalian tissue-parasite, together with *Toxoplasma*, *Hammondia* and *Sarcocystis*, recently found to produce isospora-type oocysts.

Significance to Biomedical Research and the Program of the Institute

Information on these parasites is of biologic interest, and also of medical interest because of their similarities to *Toxoplasma* and *Sarcocystis*, both of which are known to infect man.

Proposed Course of the Project

This project has been terminated. The life cycle of the *Besnoitia* species is being studied in detail by Dr. J. K. Frenkel.

Keyword Descriptors

Besnoita, cat, coccidia, *Hammondia*, oocyst, life cycle, *Toxoplasma*

Publications

Wallace, G.D.: Observations on a feline coccidium with some characteristics of *Toxoplasma* and *Sarcocystis*. Zeitschrift fUr Parasitenkunde. In press.

Wallace, G.D. and Frenkel, J.K.: *Besnoitia* sp. (Protozoa, sporozoa, Toxoplasmatidae): Recognition of cyclic transmission by cats. Science. In press.

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RML

LABORATORY OF IMMUNOLOGY

SUMMARY I

Project No.

Z01 AI 00030-07 - Antigen Recognition and Activation of Immuno-Competent Cells VII

Z01 AI 00033-07 - Lymphocyte Interactions, Receptors and Functions XIV

Z01 AI 00034-03 - Differentiation and Activation of Mouse T and B Lymphocytes XIX

Z01 AI 00035-02 - Specificity in Immune Responses XXII

Z01 AI 00036-10 - Immunoglobulin Genetics: Regulation of Gene Expression and Lymphoid Differentiation XXVII

Z01 AI 00037-08 - Immunogenetics of Mouse Immunoglobulin and Genetic Control of Antibody Responses XXXIV

Z01 AI 00038-03 - Structural and Activity Studies on Immunologically Important Cells and Proteins XL

Z01 AI 00039-01 - Tumor Immunology in Inbred Guinea Pigs XLIII

Z01 AI 00040-01 - Genetic Control of Immunocompetent Cell Interactions XLVII

Z01 AI 00041-01 - Interactions of Lymphocytes in the Immune Response LIV

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PHS-NIH
Summary Statement
Office of the Chief
Laboratory of Immunology
July 1, 1974 through June 30, 1975

The past year represented one of substantial scientific accomplishment on the part of members of the Laboratory of Immunology, which is reflected both in the publications from the Laboratory and in the participation of Laboratory staff in a variety of national and international meetings. During the past year one associate, Dr. Michael Karpf, completed his training in the Laboratory as did Dr. Shlomo Z. Ben-Sasson, a Visiting Fellow, and Dr. Hartmut Ruhl, a Guest Worker. Each of these scientists made substantial scientific accomplishments during his training period. Three new associates, Dr. Benjamin Schwartz, Dr. Theodore Werblin and Dr. Steven Siegel, joined the Laboratory and Dr. Ronald Schwartz transferred from the Immunology Branch, NCI, to this Laboratory. In addition, Dr. Donald Mosier and Dr. Myron Waxdal became permanent members of the Laboratory during the past year.

Laboratory scientists play increasingly important roles in the national and international immunologic community. Thus many serve on editorial boards of important journals: Dr. Paul is section editor for cellular immunology for the Journal of Immunology and Dr. Mage and Dr. Green are associate editors of this journal. Ms. Lieberman, Dr. Mage and Dr. Paul are advisory editors of Immunochemistry. Dr. Mage is a member of the editorial board of Federation Proceedings and Dr. Green and Dr. Inman are members of the board of reviewers of this journal. In addition, Dr. Paul is an associate editor of the Journal of Experimental Medicine, Immunogenetics, the Scandanavian Journal of Immunology and Immunologic Communications and Dr. Green is a member of the editorial board of Clinical Immunology and Immunopathology. Dr. Green is also editing a book on Mechanisms in Tumor Immunology for John Wiley and Sons.

Dr. Paul chaired symposia at the Ninth Leucocyte Culture Conference and at the Annual meeting of the American Association of Immunologists and chaired sessions at the International Workshop on Mitogens and at the Gordon Conference on Tumor Immunology. He was an invited lecturer at the American Association of Immunologists Summer Course and at the Course sponsored by the Center for Immunology at the State University of New York at Buffalo. In addition, he presented major talks at the International Symposium on Suppressor Cells, the symposium on Structure and Function at the Lymphocyte Surface at the national meeting of the American Federation for Clinical Research and at the Miles Symposium. He served as a member of the Travel Awards Committee of the American Association of Immunologists, is a member of the Transplantation and Immunology Advisory Committee of NIAID and the International Fellowship Review Panel of the Fogarty International Center.

Dr. Mage was co-chairman of a workshop at the Second International Congress of Immunology, is a member of the Allergy and Immunology Study Section of the Division of Research Grants, NIH and is a member of the American Association of Immunologists Committee on the Status of Women.

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Ms. Lieberman received a DHEW Superior Service Award, served as a Visiting Professor in the Department of Microbiology of the University of Miami School of Medicine and as a Visiting Scientist at the Wiezmann Institute of Science in Rehovoth, Israel. She was a member of the Program Committee of the American Association of Immunologists.

Dr. Green was the Chairman of a Workshop at the Second International Congress of Immunology and was an invited participant at an International Meeting on Membrane Receptors on Lymphocytes in Paris. He served as a member of the Program Committee of the American Association of Immunologists.

Dr. Mosier presented major talks at the Ninth Leucocyte Culture Conference, the International Symposium on the Role of Suppressor Cells in Immunity and at the International Workshop on Mitogens. Dr. Shevach presented lectures at the national meeting of the American Society of Hematology, the Ninth Leucocyte Culture Conference and at the American Association of Immunologists symposium on Cellular and Soluble Factors in the Regulation of Cellular Interaction. Dr. Waxdal presented a major talk at the International Workshop on Mitogens and Dr. Inman was an invited speaker at the First International Conference on Solid-Phase Methods in Protein Sequence Analysis. In addition, members of the laboratory presented seminars at major universities throughout the country.

The Laboratory of Immunology has as its basic goal the exploration of fundamental mechanisms in cellular immunology, immunogenetics, immunochemistry, immunopathology and tumor immunology. To these ends, studies of the ontogeny and heterogeneity of lymphocytes, of the nature and function of antigen-binding membrane receptors of lymphocytes, and of the mechanisms of tolerance induction and lymphocyte interactions are being actively pursued. Major efforts are being exerted to elucidate the nature, genetics and mode of function of specific immune response gene products. Mechanisms of cell mediated cytotoxicity, of the nature of the genetic and chemical control of immunoglobulin synthesis and variability and of the activation of immunocompetent cells are being intensely studied.

A FAMILY OF CROSS-REACTIVE IDIOTYPES EXPRESSED ON LEVAN-BINDING MYELOMA PROTEINS AND ANTIBODIES

The understanding of the mechanisms by which the information for the enormous number of distinct antibody molecules is organized within the genome of vertebrates and the means by which this information leads to the construction and assembly of antibodies is one of the principal challenges of modern molecular immunology. One powerful tool in this study is to use unique antigenic determinants associated with the Fab regions of mouse myeloma proteins as genetic markers. Through the use of such procedures, linkage of genes controlling the variable and constant portions of immunoglobulin (Ig) heavy (H) chains has previously been shown. This year, a series of BALB/c mouse myeloma proteins capable of binding levan have been demonstrated to bear both idiotypes unique to each protein and one or more idiotypes shared by other levan-binding myeloma proteins but not found on a large series of non-levan binding proteins. Initial studies of a large number of mouse strains immunized with levan indicate that anti-levan antibody binding molecules also bear these shared idiotypic determinants and that the expression of these idiotypes is controlled by a gene (or series of genes) linked to the genes controlling H chain constant regions. This delineates a genetic sub-region in the region controlling Ig structure devoted to molecules capable of binding levan and further structural and genetic analysis of this region promises to yield important insights into the mechanisms by which the variability of Ig molecules is determined and through which gene products are assembled into antibody molecules. (Lieberman and Humphrey, LI; Potter, Mushinski and Vrana, LCBGY, NCI).

ACTIVATION OF LYMPHOCYTES BY MITOGENIC LECTINS: MEMBRANE SITES TO WHICH LECTINS BIND

One of the critical events in immunobiology is the lymphocyte activation which occurs as a result of the interaction of antigens with surface receptors. One valuable model system to study this activation is the use of certain plant lectins and bacterial products which activate all lymphocytes of a given class or sub-class, irrespective of the nature of their specific antigen-binding receptors. A series of plant lectins have been studied in terms of the lymphocyte surface structures to which they bind. The lectins employed were concanavalin A, the family of pokeweed mitogens (Pa 1 - Pa 5), lentil lectin and wheat germ agglutinin. By radiolabelling studies, it has been shown that the principal surface molecules to which these lectins bind are a small series of glycoproteins including the H-2D and H-2K antigens and the I-region antigens, as well as surface immunoglobulin. Although it is not yet clear which, if any, of these molecules is the critical membrane "receptor" for lectins the finding that products of the major histocompatibility complex are among the principal glycoproteins to which lectins bind is particularly striking in view of the major role these molecules are now known to play in the induction and regulation of specific immune responses. (Waxdal and Nillson, LI).

ABNORMAL DISTRIBUTION IN AMOUNT OF SURFACE IMMUNOGLOBULIN PER LYMPHOCYTE IN MICE WITH AN X-LINKED B CELL DEFECT

CBA/N mice have an X-link genetic defect which prevents them from making immune responses to a series of thymus-independent antigens, although they respond normally, or near normally, to thymus-dependent antigens. This genetically determined defect provides an excellent situation in which to study the factors critical to the activation of thymus-independent (B) lymphocytes. One important question is whether this defect might reflect some abnormality in the surface antigen-binding receptors of B lymphocytes of CBA/N mice. To study this point, the relative amounts of fluorescein labeled anti-immunoglobulin (Ig) bound by individual B lymphocytes was measured using the fluorescence activated cell sorter. It was found that spleen lymphocyte-populations from adult animals of normal strains have a characteristic pattern of distribution of amounts of surface immunoglobulin per cell with a marked peak of cells which have low to intermediate amounts of surface Ig. Spleen cells from adult CBA/N mice have markedly diminished numbers of cells with low to intermediate amounts of surface Ig, although they have normal numbers of B lymphocytes with large amounts of surface Ig. In many respects, their pattern resembles that of B lymphocytes from bone marrow of normal adult mice or spleen cells from neonatal mice. The data are most consistent with CBA/N mice having a maturational arrest in the line of B cells capable of responding to T-independent antigens. (Scher, NMRC, Sharrow, IB/NCI, Paul, LI/NIAID).

FAILURE OF IN VITRO TOLERANCE INDUCTION IN CBA/N MICE.

As noted above, CBA/N mice have an X-linked defect which prevents them from responding to T-independent antigens. This is true not only when the mice themselves are immunized but also when *in vitro* antibody responses are measured. In particular, spleen cells from CBA/N and male progeny of (CBA/N x DBA/2) breedings fail to develop cells secreting anti-trinitrophenyl TNP-antibody upon immunization with TNP-AECM-Ficoll although they respond normally to the thymus-dependent antigen, TNP-sheep red blood cells (SRBC). It has been shown that the DNP derivative of the copolymer of *D*-glutamic acid and *D*-lysine (DNP-*D*-GL) induces a state of specific immunologic tolerance *in vitro* and *in vivo* in normal mice which is characterized by lack of responsiveness to both TNP-AECM-Ficoll and TNP-SRBC. However, when spleen cells from F_1 male progeny of (CBA/N x DBA/2) crosses are exposed to DNP-*D*-GL at concentrations as high as 100 μg/ml, their responsiveness to TNP-SRBC is unimpaired, indicating that DNP-*D*-GL fails to induce tolerance in these mice. This result provides strong evidence that specific B lymphocyte tolerance to DNP-*D*-GL is an active process and that the defect in CBA/N mice is such that they neither respond to nor are tolerized by T-independent antigens and tolerogens. (Cohen and Mosier, LI/NIAID; Scher, NMRI).

MACROPHAGE-LYMPHOCYTE INTERACTION IN INITIATION OF SPECIFIC T LYMPHOCYTE DNA SYNTHESIS: REQUIREMENT THAT MACROPHAGES BE DERIVED FROM GUINEA PIGS OF "RESPONDER" HISTOCOMPATIBILITY TYPE.

It has previously been shown that the activation of guinea pig T lymphocytes by specific antigens requires (or is markedly amplified by) the presentation of the antigen by macrophages. In particular, for optimal responses, the macrophages and the lymphocytes must share at least one haplotype derived from the major histocompatibility complex (MHC), suggesting that MHC gene products played critical roles in macrophage function or in macrophage-lymphocyte interactions. Furthermore, when antigen-systems, responsiveness to which is controlled by MHC-linked immune response (Ir) genes are studied, macrophages only function efficiently when they derive from a donor which possesses a responder allele at the given Ir locus. During the past year, further genetic studies have demonstrated that it is very likely the Ir genes themselves, or some extremely closely linked genes, which must function in the macrophage. Thus, some outbred guinea pigs are able to respond to the copolymer of glutamic acid and tyrosine (GT) and to low doses of DNP-guinea pig albumin (DNP-GPA). The Ir genes controlling GT (*GT*$^{+}$) and DNP-GPA (*DNP-GPA*$^{+}$) responsiveness are linked to the genes controlling strain 13 alloantigens (*13*$^{+}$). Thus, many outbred animals have one haplotype which can be tentatively written *DNP-GPA*$^{+}$, *GT*$^{+}$, *13*$^{+}$. However, some outbred animals have haplotypes which are DNP-GPA$^+$, GT$^-$, 13$^+$ and others are DNP-GPA$^-$, GT$^+$, 13$^-$. It is found that lymphocytes from DNP-GPA$^+$, GT$^-$, 13$^+$ can be stimulated to respond to DNP-GPA by DNP-GPA-pulsed macrophages from DNP-GPA$^+$, GT$^-$, 13$^+$ donors but not by macrophages from DNP-GPA$^-$, GT$^+$, 13$^-$ donors. On the other hand, GT pulsed macrophages from DNP-GPA$^-$, GT$^+$, 13$^-$ cells activate lymphocytes from DNP-GPA$^-$, GT$^+$, 13$^-$ animals while macrophages from DNP-GPA$^+$, GT$^-$, 13$^+$ donors do not. This result strongly suggests that immune response genes or genes closely linked to them play critical roles in macrophage function. (Shevach, LI/NIAID).

GENETIC REGULATION OF COLLABORATION BETWEEN MOUSE T AND B LYMPHOCYTES.

It has recently been shown that the helper interactions between T and B lymphocytes important in the development of thymus-dependent antibody responses are most efficient when the donors of the T and B lymphocytes share at least one major histocompatibility complex (MHC) haplotype. Mapping studies suggest that the critical genes are found within the I-region of the MHC. By evaluation of the capacity of cells from congenic mice bearing MHC's derived by genetic recombination, it has been possible to localize the genetic region which must be held in common even more precisely. This was done by studying the collaborative interactions of B and T lymphocytes from B10.A (2R) and B10.A (4R) mice. These mice are similar in the K, IA and D regions of the MHC and differ in IB, IC, and S regions. Thus, the role of K, IA and D can be studied. Our results indicate that cells from B10.A (2R) and B10.A (4R) mice collaborate quite well in response to DNP-derivatives of IgG and IgA BALB/c myeloma proteins. This work, together with previous studies of Katz, *et al.*, indicates that for these two antigens at least, I-A similarity is important in collaboration. As the immune response to IgG and IgA myeloma protein is controlled by MHC-linked Ir genes, this data also can be used to analyze the cellular locus of the gene defect in this system. The results indicated that T lymphocytes from B10.A (2R) mice can make some response to IgG proteins although the B10.A (2R) mouse itself does not and B cells from the (2R) mouse respond poorly to DNP-IgG. The results suggest that this Ir gene is expressed, at least to some extent, in B lymphocytes. This does not rule out that Ir gene expression also occurs in T-lymphocytes. (Janeway, Werblin, Lieberman and Paul, LI/NIAID).

STRUCTURE OF GUINEA PIG Ia-LIKE ANTIGEN

As described in the two previous sections, products of the major histocompatibility complex (MHC) play critical roles in specific immune responses. Current evidence from both mouse and guinea pig studies is that I-region associated antigens are most important in this regard. This is particularly interesting as the I-region is the gentic region of the MHC in which the specific immune response genes are found. In last year's annual report, studies of guinea pig I-region associated antigens by surface iodination with lactoperoxidase were described. These studies indicated that the Ia antigens "2" and "13" were molecules of ~ 25,000 daltons in size. This year, further efforts have been made using internal labeling procedures and a variety of more recently described anti-Ia sera. These results indicate that I-region antigens are on separate molecules from other MHC antigens (i.e., B, C, D, etc.) and that at least two distinct groups of I-region

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molecules exist in extracts from lymphoid cells of inbred guinea pigs. Furthermore, studies using ^{3}H-leucine and ^{3}H-Fucose as precursors indicate that the I-region antigens are glycoproteins and that they consist of two polypeptide chains linked by disulfide bonds. Thus, when isolated without reduction, a molecule of ~ 58,000 Daltons in size is obtained which upon reduction yields molecules of 33,000 and 25,000. The latter appears to be the polypeptide chain identified by surface iodination. This double chain structure for Ia is particularly interesting in view of the possibility that immune recognition functions are associated with this molecule. Further studies of the chemical nature, heterogeneity and antigen-specificity of Ia antigens are underway. (B. Schwartz, Shevach and Paul, LI/NIAID).

RECEPTOR FOR C3b IN THE HUMAN RENAL GLOMERULUS

In the course of studies of the nature of infiltrating lymphocytes present in the kidney in certain human renal diseases, reagents capable of detecting receptors for the activated fragments of the third component of complement (C3) were used as such receptors are present on many human B lymphocytes. The striking observation was made that such reagents localized to the glomerulus and were found there whether lymphocytes were present or not. This suggested that a cell type within the glomerulus had a receptor for C3. This is a particularly important finding in terms of the pathogenesis of immune complex renal disease, in which C3 is localized in the glomerulus and is thought to play an important role in tissue damage. Further studies indicate that the C3 receptor in the glomerulus is specific for the C3b fragment and that it is present on the surface of endothelial cells. The relationship of this receptor to human immune complexes diseases are now being studied. (Gelfand, Georgetown University School of Medicine, Green, LI/NIAID and Frank, LCI/NIAID).

Project No. Z01 AI 00030-07 LI
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Antigen Recognition and Activation of Immunocompetent Cells

Previous Serial No.: 140

Principal Investigator: Dr. William E. Paul

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Dr. Michael Gelfand, Department of Med. Georgetown University Med. Center

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to	Guest Workers	TOTAL
Total	6 2/12			6 2/12
Professional	4 2/12			4 2/12
Other	2			2

Keyword Descriptors: T lymphocyte, B lymphocyte, receptors, lymphocyte activation, cell membrane immunoglobulin, CBA/N mice, allograft prolongation.

Project Description:

The general goal of this project is to delineate the mechanisms involved in control of lymphocyte activation, particularly by the study of membrane events occurring in antigen recognition and by the investigation of how such recognition initiates the response of the cell. To this end, studies of a functional and structural nature have been undertaken on both thymus-dependent (T) and thymus-independent (B) lymphocytes.

Project Subtitle I: Development and utilization of an assay for activation of mouse T lymphocytes

Although much is known about the interactions of mouse T and B lymphocytes and about the activation of mouse T lymphocytes capable of mediating mixed lymphocyte and cytotoxic reactions which are directed principally at histocompatibility antigens, little information is available about direct T lymphocyte activation by conventional antigens because of the lack of a simple assay procedure in the mouse. The method which has been most successful in guinea pig and human systems has been the initiation of T lymphocyte DNA synthesis by antigens but, for a variety of technical reasons, this procedure has not been regularly successful in mouse systems. Because of the relatively advanced understanding of the genetics of the mouse immune system and of the major histocompatibility complex (MHC) of the mouse, a direct T lymphocyte activation assay could be extremely useful. In the course of the past year, such an assay has been developed. It involves the immunization of inbred mice with antigen emulsified in complete Freund's adjuvant and the induction, 10 days to six weeks later, of a peritoneal exudate using thioglycollate. Peritoneal exudate cells are harvested four to five days later and the lymphocytes are partially purified by sequential adherence to glass dishes and passage over nylon wool columns. The cells which are obtained are highly enriched in T lymphocytes and contain very few (~2%) B lymphocytes. When cultured in microtiter dishes in EHAA medium supplemented with fetal calf serum, these cells give marked proliferative responses to antigens to which the donor has been primed. The responses are a function of T lymphocytes as they can be abolished by treatment with anti-Thy 1.2 serum and complement and they demonstrate conjugate specificity in hapten-carrier systems. B lymphocytes make little contribution to the proliferative response as the great majority of all transformed cells bear the Thy 1.2 antigen.

Using this technique, the requirement for sharing of MHC antigens in macrophage-T lymphocyte interactions and the role of antigens encoded in the I region of the MHC in antigen recognition have been studied. Work now in progress indicates that maximal macrophage presentation of antigen to T lymphocytes occurs only if the two cell types share MHC antigens and that antisera directed at I region (but not D and K region) antigens block T cell activation, especially by antigens responsiveness to which is determined by MHC linked immune response (Ir) genes. These studies thus confirm previous work from the Laboratory of Immunology, carried out in in vitro guinea pig systems. However, because of the superior understanding of mouse genetics, it is anticipated more detailed investigation of the mechanism and significance of these findings can be carried in mouse systems than would be possible in guinea pigs. Such information promises major insights into the process by which T lymphocytes recognize, and are activated by, antigen.

Project Subtitle II: Antigen-binding by guinea pig thymocytes

The studies described in the previous project are aimed at understanding T lymphocyte antigen-recognition by investigating the requirements for lymphocyte activation. The alternative approach, which is employed in the studies described in this sub-title, is to study the direct binding of antigen to T lymphocytes. Unfortunately, any individual antigen is bound by only a small fraction of cells and many more B lymphocytes than T lymphocytes can be demonstrated to bind a given antigen. Consequently, it is very difficult to investigate specific antigen-binding by T lymphocytes in mixed cell populations. One approach is to measure antigen-binding by thymocytes as very few B lymphocytes (≤ 1%) are found in this cell population. The studies were performed by preparing thymocyte suspensions, chilling them to 4°C, mixing them with ^{125}I-labeled 2,4-dinitrophenyl guinea pig albumin (DNP-GPA) at 0.2 μg/ml, washing the cells thoroughly, preparing radioautographs and then measuring the number of thymocytes which have bound detectable amounts of DNP-GPA, as judged by silver grains over the cell. In general, from 40 to 60 thymocytes per 100,000 bind DNP-GPA and this binding can be inhibited by excess non-radioactive DNP-GPA although not by non-radioactive DNP conjugates of other proteins. This is consistent with the specificity requirements for activation of T lymphocytes and differs from the binding properties of the receptors of B lymphocytes which bind DNP-GPA. The latter are inhibited equally well by a variety of DNP-conjugates. Antisera directed at antigens encoded within the I region of the guinea pig MHC cause partial inhibition of binding of DNP-GPA to guinea pig T lymphocytes. This is in keeping with the previously demonstrated capacity of such sera to block activation of T lymphocytes by antigen and suggests that I-region antigens have an important role in antigen-recognition by T lymphocytes. One surprising result was the capacity of two separate anti-immunoglobulin (Ig) sera, one directed at multiple Ig determinants and the other specific for Fab determinants, to block antigen-binding by thymocytes. This is in contrast to our failure to block T cell activation with four separate anti-Ig sera. Further studies on this subject are in progress. If these results can be confirmed and extended, they would strongly indicate that lymphocyte membrane molecules with Ig determinants and I-region determinants both play crucial roles in antigen-recognition by, and activation of, guinea pig T lymphocytes.

Project Subtitle III: The use of mice with an X-linked B lymphocyte defect in the study of B lymphocyte activation

Substantially more progress has been made in the study of the antigen-binding receptors of B lymphocytes than of T lymphocytes. However, the mechanism by which the binding of antigen to receptors of B lymphocytes causes the activation of these cells is still unknown although at least three distinct types of theories have been proposed to explain this activation. Recently, Baker and his colleagues have reported that the NIH sub-line of the CBA strain of mice (CBA/N) fails to respond to type III pneumococcal polysaccharide. Further investigation has shown that these mice fail to respond to all T-independent antigens thus far tested, including lipopolysaccharides, poly-inosinic·poly-cytidilic acid (poly I·C) and 2,4-dinitrophenyl (DNP)-lysyl-Ficoll. In each case, the failure to respond has been shown to be an X-linked trait. Because of the enormous potential value of these mice, an extensive series of studies of the immunologic system of CBA/N mice has been initiated. The following findings have been established:

1. CBA/N mice make essentially normal responses to T-dependent antigens including DNP-protein conjugates, suggesting their B cell defect is limited to the T-independent system.

2. CBA/N mice make normal T cell responses, as measured in a wide variety of tests.

3. CBA/N mice have diminished numbers of splenic B lymphocytes. The fraction of B lymphocytes with surface Ig is approximately half that of normal strains. Measurement of numbers of cells capable of binding DNP-proteins indicates that, per B cell, these occur in normal numbers confirming that the failure of CBA/N mice to respond to T-independent antigens such as DNP-Lys-Ficoll is not a function of the absence of cells bearing receptors for such antigens.

4. The B lymphocytes possessed by CBA/N mice differ from those of normal mice in that they yield substantially smaller DNA synthesis responses to lipopolysaccharide and poly I-C, which are B lymphocyte mitogens. Their responses to T lymphocyte mitogens are normal. In addition, the distribution in amount of surface Ig per lymphocyte is abnormal in that the spleens of CBA/N mice have markedly diminished numbers of B lymphocytes with small to intermediate amounts of surface Ig while the number of CBA/N B cells with large amounts of surface Ig is essentially normal. Finally, studies of surface immunoglobulin by lactoperoxidase catalyzed iodination of membrane molecules indicate the CBA/N mice have a substantial diminution in the amount of an immunoglobulin heavy chain which has been proposed as the mouse equivalent of human δ chain.

5. Each of these defects is X-linked in that they are seen in the CBA/N mouse and also in (CBA/N x DBA/2)F_1 male mice while F_1 female mice are normal. Moreover, transfer of very small numbers of F_1 female B lymphocytes or bone marrow cells to F_1 male recipients replenished their response to T-independent antigens establishing that the defect is intrinsic to the B cell line.

These results can be interpreted in two ways. In one, a qualitative abnormality in the B cell is proposed which prevents these cells from responding to T-independent antigens. According to this hypothesis, one of the three principal abnormalities (i.e., low responses to B-mitogens, diminished numbers of B cells with intermediate amounts of surface Ig, and diminished amounts of surface IgD might be the critical defect of CBA/N B cells. This would in turn suggest that normal activation of B cells by T-independent antigens critically depended upon this particular cell function.

The alternative possibility, which is made more likely by the multiple defects in the B cells of CBA/N mice, is that a subpopulation of B lymphocytes specialized to react to T-independent antigens is lacking. This population would be expected to have the characteristics of marked responsiveness to B-mitogens, intermediate amounts of surface Ig and a relative predominance of IgD.

Further studies to attempt to choose definitively between these two possibilities are in progress. The delineation of the defect in these mice should provide critical insights into B cell activation and should be helpful in understanding immunologic defects in human X-linked immunodeficiency diseases, especially Weskott-Aldrich syndrome which has several similarities to the constellation of defects expressed in the CBA/N mice.

Project Subtitle IV: Surface immunoglobulin of human cord blood lymphocytes.

In the last several years, a new technique has been introduced which allows radiochemical identification and analysis of cell membrane components by catalytic iodination of living cells. Lactoperoxidase is employed for this purpose and the isotope used is generally ^{125}I. Because the enzyme is excluded from the cells, iodination is limited to molecules on the outer surface of the plasma membrane. We have previously used this method to characterize guinea pig cell membrane histocompatibility antigens. This year we extended these studies to an analysis of cell membrane immunoglobulin of human and animal cells with particular interest in cell membrane IgD. We had previously demonstrated by immunofluorescence that, although IgD was a very rare component of the serum, it was present on a substantial fraction of Ig bearing lymphocytes of adult peripheral blood. Subsequently, Rowe and his colleagues found that ~ 20% of human cord blood lymphocytes fluoresced when exposed to fluorescein labeled anti-IgD antibody. More recently, Melcher et al. and Abney and Parkhouse, using lactoperoxidase catalyzed iodination, have described an immunoglobulin H chain on adult mouse spleen and lymph node cells which was neither μ, α or γ and which they proposed to be the mouse analog of δ. We have repeated these studies and confirm the finding of an Ig H chain with a mobility on SDS-polyacrylamide gel electrophoresis distinct from μ, γ and α. However, we are unable to identify an Ig H chain with this mobility on rabbit or guinea pig spleen and lymph node cells or on human cord blood lymphocytes. Moreover, we have positively identified the δ H chain on human cord blood lymphocytes as having an electrophoretic mobility indistinguishable from human μ chain. This raises the possibility that the newly described mouse H chain may not be a δ analog.

In the course of these studies, we found that polyvalent anti-human Ig antibodies and a specifically purified human anti-γ antibody precipitated a group of 3 molecules which were not precipitated by anti-κ chain antibodies. The nature of these molecules is unknown but one of them appears to be the principal molecule in the membrane of the cord blood lymphocyte which is labeled by ^{125}I. The capacity of anti-Ig to precipitate this cell membrane component is not removed by absorption with either human cord blood serum bound to Sepharose or a Sepharose conjugate of the serum of patients with absolute agammaglobulinemia. Our current view is that this membrane component cross reacts with γ chain, probably through common carbohydrate components. Further studies to identify this molecule are in progress.

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Project Subtitle V: The role of regulatory T lymphocytes in Prolongation of skin allograft survival by anti-Thy 1 serum.

Thy 1 (Θ) is an alloantigen which appears on the surface of thymocytes and T lymphocytes of mice in two allelic forms, Thy 1.1 and Thy 1.2. Administration of Thy 1.2 to BALB/c mice ($H-2^d$, Thy 1.2) prolongs the period required for these mice to reject C57BL/6 ($H-2^b$, Thy 1.2) skin from 9.5 days to ~ 13 days depending upon the preparation and dose of anti-Thy 1.2 employed. Five separate anti-Thy 1.2 preparations all show activity and a dose of 0.5 ml of a 1:128 dilution is effective. Using 0.5 ml of a 1:16 dilution, the graft prolonging effect could be demonstrated up to two weeks later. The activity was demonstrated to be a function of the anti-Thy 1.2 content of the serum as it could be specifically removed by homogenates of C3H brain, a tissue rich in Thy 1.2, but not by homogenates of AKR brain, which contains the allelic Thy 1.1 antigen. Finally, anti-Thy 1.1 serum prolonged survival of C57BL/6 (Thy 1.2) allografts on AKR (Thy 1.1) mice indicating that the effect of the serum was exerted principally on host and not donor tissue.

The effect of anti-Thy 1.2 appears not to be mediated by depletion of cytotoxic effector T lymphocytes for a variety of reasons. Adult thymectomy three weeks or more prior to treatment with anti-Thy 1.2 and grafting abolishes the graft prolonging effect of anti-Thy 1.2 administration although it has no effect on graft survival in untreated mice. Similarly, administration of small doses of anti-thymocyte serum (ATS) abolishes the graft prolonging effect of anti-Thy 1.2. These results indicate that a cell which can be eliminated by adult thymectomy or ATS treatment is required for the graft prolonging effect of anti-Thy 1.2. Moreover, spleen cells and thymocytes from anti-Thy 1.2 treated BALB/c mice, when transferred to untreated syngeneic recipients, cause prolonged allograft survival (up to 15.5 days). The most likely explanation of this result is that a population of regulatory (suppressor) T lymphocytes is activated by anti-Thy 1.2 treatment and these cells are responsible for diminishing the response to a skin allograft. The use of such activation of regulatory T lymphocytes might be a more physiologic strategy to obtain immunosuppression in clinical situations than those procedulres now employed.

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Project No. Z01 AI 00033-07 LI
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Lymphocyte Interactions, Receptors and Functions

Previous Serial No: None

Principal Investigator: Dr. Ira Green

Other Investigators: Project Subtitle I:
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Dr. John Coe, Rocky Mountain Laboratory, Hamilton, Mont.

Project Subtitle II:
Dr. Irwin Scher, National Naval Medical Center, Bethesda, Md.

Cooperating Units: Hematopathology Section, Laboratory of Pathology, NCI, Laboratory of Clinical Investigation, Laboratory of Viral Disease, NIAID, Rocky Mountain Laboratory, NIAID, Naval Medical Research Laboratory, Bethesda, Md.

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to	Guest Workers	TOTAL
Total	1-6/12			1-6/12
Professional	1			1
Other	6/12			6/12

Project Subtitle I: Determination of the origin of mononuclear cells in human pathological conditions

Keyword Descriptors: Lymphocytes, mononuclear cells, receptors, immune response genes

Mononuclear cells that participate in the immune response can be characterized by virtue of their having specialized membrane receptors, surface antigens and functions. These cells participate in a variety of immunopathologic conditions and also malignant transformations of these cells occur.

The determination of the presence of specialized surface features on mononuclear cells allows for a positive identification of a mononuclear cell of unknown origin. These techniques can be performed on cells in suspension or in sections of tissue. Two disease entities that have been recently investigated in collaborative efforts by our laboratory are childhood lymphosarcoma (CLSA) and lymphomatoid papulosis.

Six patients with CLSA have been examined. The malignant cells from one patient had T cell markers, while those from two patients had B cell markers. In addition, one patient had cells with markers of both B cells and T cells and the cells of two patients had no detectable markers. These results suggest that this disease entity is quite heterogeneous. A positive clinical correlation was made between the presence of the markers and the development of central nervous system disease. The finding of T cell markers in certain patients with CLSA suggest that the disease process in these patients may be quite similar to those patients with acute lymphocytic leukemia that also have cells with T cell markers.

Lymphomatoid papulosis (LP) is a rare benign dermatological disease characterized by a lymphocytic infiltrative process with some clinical and pathologic resemblence to the malignant disease, mycosis fungoides. It has already been established that the cells of mycosis fungoides have characteristics of T cells. Cells obtained from the lesions of two patients with LP demonstrated surface markers characteristic of thymus derived (T) cells. These studies strongly suggest that these two disease entities are very closely related; the reasons that the disease in one instance pursues a benign course whereas in the form of mucosis fungoides a malignant course is observed remains to be explained.

The next conditions in which studies were attempted were in immunopathological diseases in which a mononuclear cell infiltrate is a prominent feature.

A large body of immunological literature exists that describes a variety of in vitro mononuclear cell mediated cytotoxicity. The mechanism and precise cell types involved in these in vitro cytotoxic reactions are under intensive investigation because it is felt that these mononuclear cells have important in vivo roles in autoimmune disease, defense against infection and parasitic disease, and in the host response to malignancy. There is, however, a surprising lack of information concerning the precise mononuclear cell types in in vivo pathological situations. Information in this latter area would allow a better correlation between the in vitro phenomenology and the in vivo pathology.

For example, mononuclear cells are commonly found in the vicinity of malignant tumors; breast carcinoma and melanoma are two examples. Also, in the latter instance there is evidence the melanoma cell or its products are antigenic in the host.

We therefore undertook a study to identify the mononuclear cell types found in the vicinity of several types of melanotic lesions. Tissue sections were prepared and the adherence of red cell reagents were used to identify the type of mononuclear cell present in the lesion. Also, mononuclear cells were teased out of the lesion and reacted with red cell reagents.

In six primary melanomas and in halo nevi, the cells in the lesions appeared to be T lymphocytes. However, in metastatic melanoma lesions the cells were principally histiocytes. In one patient with a giant benign pigmented nevus the infiltrating cells appeared to be B lymphocytes.

All of these three cell types are capable of mediating *in vitro* cytotoxic reactions.

It is hoped that further studies of this type will be useful in understanding the role of these cells in defense against malignancy. For example, the effects of immunotherapy in this disease can be correlated with both the *in vitro* effects of mononuclear cells from these patients on tumor target cells, and simultaneously the types of mononuclear cells in the lesions.

In the course of other studies designed to establish the nature of mononuclear cells in kidney sections from patients with interstitial nephritis it was observed that indicator sheep red blood cells (SRBC) bearing the third component of complement (C3) adhered to the renal glomerulus. This observation suggested that some structure within the glomerulus had a C3 receptor. This finding is of considerable importance since it may explain the fact that in many renal diseases, immune complexes bearomg C3 become preferentially trapped within the glomerulus. This trapping of complexes within the glomerulus may then lead to damage to the basement membrane. The structure within the glomerulus that has the C3 receptor is presently being investigated.

One other cell type that has been studied are cells from two SV_{40} induced lymphomas in the hamster. These tumors were induced by G. Diamandopoulos, Harved Medical School, Boston, Mass., by the intravenous injection of weanling hamsters with SV_{40} virus. Both these lymphoid tumors have 7Sγ2 globulin on their surface indicating they are of B cell origin. In addition, one of these tumors had an idiotypic immunoglobulin. These tumors may be useful for further study of the mechanism of SV_{40} virus leukemogenesis (for example, do B cells have a receptor for SV_{40} virus). Also the idiotypic immunoglobulin present on the surface of one of these tumors could serve as the target for an immunotherapeutic attack.

In summary, these studies represent an attempt to more precisely identify the nature of both malignant mononuclear cells (certain leukemias and lymphomas) and benign mononuclear cells in various immunopathologic lesions. It is hoped that this identification, as well as immunological functional studies of the cells, will provide insights into the pathophysiology of certain of these diseases.

Project Subtitle II: Use of a Synthetic Poly Amino Acid Antigen in Humans

The immune response to a large number of synthetic poly amino acid antigens, as well as natural antigens, has been shown to be controlled by autosomal dominant genes in several animal species. The genes controlling these responses (Ir genes) are within the major histocompatibility complex of the species. In man, there is a relationship between the frequency of certain diseases and the presence of certain HL-A types. The presence of Ir genes in humans linked to a particular HL-A type or closely associated MLR type may be one of the reasons for this association. Thus, susceptibility to certain human diseases would be associated with particular HL-A type because of a close linkage between immunological responsiveness and/or lack of responsiveness to a particular disease associated antigen and a particular HL-A type.

The identification of human HL-A linked Ir genes would be an important step in the support of such a concept. To investigate this problem we immunized adults with synthetic polymer composed of glutamic acid, lysine and tyrosine (GLT). The immune response in the subjects was then correlated with their HL-A type. Sixty-one individuals were immunized. Delayed skin test reactivity to this antigen was seen in 34 of 61 subjects immunized with 150 μg of GLT. No antibody to GLT was detected in these responding individuals. There was a close correlation between the _in vivo_ skin reactivity of volunteers to GLT and the ability of their lymphocytes to produce migration inhibitory factor (MIF) in response to GLT _in vitro_. However, a similar correlation was not seen when the _in vitro_ proliferative response of lymphocytes to GLT, as measured by (methyl-^{3}H) thymidine ((^{3}H)TdR) incorporation, was assayed.

HL-A typing of volunteers was performed to determine if responsiveness to GLT was correlated to HL-A type. No statistical association between any individual antigen and responsiveness was seen after correction was made for the number of individual HL-A antigens studied. No adverse effect was seen as a result of immunization in any patient.

Family studies to determine if there is a relationship between a particular haplotype and immune responsiveness to GLT are contemplated.

Finally, these studies demonstrate the safety and feasibility of using this type of antigen in humans. In the future, patients (for example, those with ankylosing spondylitis) in whom there is a highly significant association between HL-A and/or MLR type and the frequency of the disease could be immunized with such antigens. Such an approach could then directly test the hypothesis that these patients either had or lacked certain Ir genes.

RML

Publications:

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Coe, J. and Green, I.: "B" cell origin of SV40-induced hamster leukemias. J. Nat. Cancer Inst. 54: 269, 1975.

Yoshida, T., Edelson, R., Cohen, S., Green, I.: Migration inhibitory activity in serum and cell supernatant in patients with Sezary Syndrome. J. Immunol. 114: 915, 1975.

Miller, W.G., Steinberg, A.D., Green, I., Nussenzweig, V.: Complement dependent alterations in the handling of immune complexes by NZB/W mice. J. Immunol. 114: 1166, 1975.

Ahmed, A., Edelson, R., Strong, D., Knudsen, R., Woody, J., Sell, K. and Green, I. *In vitro* cellular reactivity of lymphocytes from patients with chronic T-cell leukemia. Clin. & Exptl. Immunol. In press.

Scher, I., Berning, A.K., Strong, D.M. and Green, I. The immune response to a synthetic amino acid terpolymer in man: Relationship to HL-A type. J. Immunol. In press.

Edelson, R., Hearing, V., Dillon, L., Edelson, E., Frank, M. and Green, I. Identification of cell populations of mononuclear cells in melanocytic lesions. Clin. Immunol. & Immunopath. In press.

Project No. Z01 AI 00034-03 LI
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Differentiation and Activation of Mouse T and B Lymphocytes

Previous Serial Number: 144

Principal Investigator: Dr. Donald E. Mosier

Other Investigators:
Dr. P.L. Cohen, LI/NIAID
Dr. I. Zitron, LI/NIAID
Dr. I. Scher, Naval Medical Res. Center
Dr. S. Cross, Micro. Assoc.
Dr. R. Tigelaar, LMI/NIAID
Dr. W.E. Paul, LI/NIAID

Cooperating Units: LMI/NIAID, Naval Medical Research Institute, National Naval Medical Center, Bethesda, Md., Microbiological Associates, Bethesda, Md.

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to	Guest Workers	TOTAL
Total	3			3
Professional	2			2
Other	1			1

Project Description:

Keyword Descriptors: T-Cell, B-Cell, Activation, Tolerance, Suppressor Cells

The major emphasis of work in this laboratory has continued to be 1) differentiation of T and B lymphocytes during ontogeny including identification of subpopulations of cell lines, and 2) requirements for activation of mouse B lymphocytes for antibody production *in vitro*. Several projects overlap both these areas; e.g. one new finding of importance was that spleen cells from newborn animals contain B lymphocytes fully capable of activation to antibody synthesis by both T-dependent and T-independent antigens but that such activation is impaired by excess numbers and/or function of suppressor T lymphocytes (Mosier and Johnson, J. Exp. Med. 141: 216, 1975). A new model for T cell depletion in neonatal mice has been developed by Dr. Philip Cohen (LI) working in collaboration with Dr. Sue Cross (Microbiological Assoc.) using a naturally-occurring herpes virus of mice, mouse thymic virus (Cohen, Cross and Mosier, J. Immunol., in press). Mouse

Thymic virus, when inoculated within 48 hours of birth, causes extensive necrosis of the thymus gland and depletion of peripheral T lymphocytes lasting 5-6 weeks. During this period of "transient thymectomy", mice are unable to produce antibody against T-dependent antigens or to initiate T lymphocyte cellular reactions such as the mixed lymphocyte reaction. The subpopulation of cells capable of suppressing B lymphocyte activation appears to survive acute virus infection, however, suggesting it may be a separate T cell line.

The direct activation of B cells has been extensively studied using DNP-Ficoll and DNP-D-glutamic acid-D-lysine (DNP-D-GL). The first a powerful T-independent antigen *in vitro* (Mosier, Johnson, Paul McMaster, J. Exp. Med. 139: 1354, 1974) and the second a T-independent tolerogen *in vitro*. Dr. Ian Zitron (LI) has worked extensively on the ability of DNP-Ficoll to activate precursor and memory B cells and the heterogeneity of the antibody produced. DNP-D-GL has been found to behave as an immunogen rather than a tolerogen when insolubilized on macrophages or agarose beads suggesting physical presentation is a major factor in determining the outcome of antigen-lymphocyte interaction. In collaboration with Dr. Philip Cohen (LI) and Dr. Irwin Scher (Naval Medical Research Institute), the B cell activation defect in CBA/N mice is being studied. This mutant subline has an X-linked trait which prevents responses to T-independent, but not T-dependent, antigens. Spleen cells from such mice have been found to be resistant to tolerization by DNP-D-GL suggesting 1) that tolerization is an active process analogous to antibody formation, and 2) that the B cells in CBA/N mice responding to T-dependent antigens are unique in their resistance to tolerance induction. B lymphocytes may belong to distinct subpopulations, e.g. one responding to T-dependent antigens and another responding to T-dependent antigens. The CBA/N cells, when stained with fluorescent anti-immunoglobulin reagents, have a unique intensity of staining as detected by the fluorescence activated cell sorter. Using similar techniques, a physical separation procedure has been found to give a distinct population analogous to CBA/N B cells. This technique gives promise of a means to directly obtain a purified B cell subpopulation.

Another model system for *in vitro* activation of B cells and T cell regulation of such activation has been developed in collaboration with Dr. Robert Tigelaar (LMI, NIAID) using pneumococcal polysaccharide type III (SIII) as antigen. Regulation of SIII antibody responses appears to involve both antibody and T cell modulation, but the classical *in vivo* system for low dose SIII suppression seems to involve mainly antibody-mediated suppression.

The goals of this research are to better understand the regulation of immune responsiveness at the cellular level in the neonatal and adult animal. Such understanding would provide a rational basis for regulating immune responsiveness to specific antigens where such regulation is desirable, e.g. in autoimmunity, graft rejection, and neonatal immunization.

PUBLICATIONS:

Mosier, D.E. and Cohen, P.L.: Ontogeny of mouse T lymphocyte function. Fed. Proc. 34: 137, 1975.

Paul, W.E., Karpf, M. and Mosier, D.E.: Activation of and tolerance induction of DNP-specific B cells; Analysis with three distinct DNP-carrier conjugates. In Immunological Tolerance: Mechanisms and Potential Therapeutic Applications (Ed.) D.H. Katz and B. Benacerraf. Academic Press, New York, 1974, pp. 141-158.

Simpson, E., O'Hopp, S., Harrison, M., Mosier, D.E., Melief, K. and Cantor, H.: Immunological disease induced by injecting F_1 lymphoid cells into certain parental strains. Immunology 27: 989, 1974.

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Mosier, D.E.: The role of macrophages in the specific determination of immunogenicity and tolerogenicity. In Nelson, D.S. (Ed.): Immunobiology of Macrophages. New York, Academic Press, 1975, in press.

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Mosier, D.E. and Johnson, B.M.: Ontogeny of mouse lymphocyte function. II. Development of the ability to produce antibody is modulated by T lymphocytes. J. Exp. Med. 141: 216, 1975.

Cohen, P.L., Cross, S. and Mosier, D.E.: Immunological effects of neonatal mouse thymic virus infection. J. Immunol., 1975, in press.

Mosier, D.E., Cohen, P.L. and Johnson, B.M.: Suppressor cells in the ontogeny of the immune response. Singhal, S.K. (Ed.): Proc. International Symposium on Suppressor Cells. Canada, MRC, 1975, in press.

RML

Project No. Z01 AI 00035-02 LI
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Specificity in Immune Responses

Principal Investigator: John K. Inman

Other Investigators: Subtitle I: Dr. Bruce Merchant, BOB, FDA
Dr. Ramesh C. Srivastava, BOB, FDA
Dr. Ettore Appella, LCB, NCI
Subtitle II: Dr. Ian Zitron, LI, NIAID
Dr. Donald E. Mosier, LI, NIAID
Dr. William E. Paul, LI, NIAID
Dr. Hillel S. Koren, NCI
Dr. John R. Wunderlich, NCI

Cooperating Units: Bureau of Biologics, FDA (Drs. Merchant and Srivastava), Laboratory of Cell Biology, National Cancer Institute (Dr. Appella), Immunology Branch, National Cancer Institute (Drs. Koren and Wunderlich)

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
TOTAL	2			2
Professional	1			1
Other	1			1

Project Subtitle I: Multispecific Properties of the Antibody Combining

Project Desceiption:

Keyword Descriptions: immune responses, immunological specificity, antibody combining regions, binding sites, multispecificity, large haptens, hemolytic plaque assays, PFC, affinity chromatography, immunoadsorption, peptide synthesis, radioiodination, amino acid sequencing, derivatization of polysaccharides, thymus-independent antigens, hapten-specific cytotoxicity

Objectives of Project Title:

The overall objective of the project is to explore the molecular basis for specificity in immune responses. Particular objectives are to study the structural and binding properties of antibody combining regions and of receptor sites on cells, to probe the extent and genetic basis of antibody diversity, and to relate the properties of various binding sites and regions with observed behavior of immune systems.

Objectives of the work under this subtitle are (1) to explore the nature and frequency of specific binding of unrelated antigenic structures by individual antibody molecules, (2) to determine the significance of this phenomenon with respect to antibody diversity, immune competence, immune surveillance and autoimmune disease, (3) to employ multispecific binding as a means for stimulating restricted immune responses and obtaining antibodies suitable for structural studies, (4) to use multispecific binding as a genetic marker for immunoglobulin variable regions.

Multispecificity is a characteristic of single antibody species rather than of the heterogeneous collection of antibody normally arising in a specific immune response. Special methods are required for studying the binding properties of individual antibodies when their prior isolation is not feasible. One successful approach has been through the use of hapten-specific hemolytic plaque assays where various soluble (and non-toxic) compounds are added to the assay medium. A fractional inhibition is sometimes observed even when these compounds bear no apparent structural similarity with either the immunizing or test hapten. Multispecificity, demonstrated by this type of inhibition, was described in last year's report for rabbit antibodies from accidentally restricted responses. This work, a collaboration with Drs. Bruce Merchant and Ramesh Srivastava (Bureau of Biologics), has been extended.

During the past year we observed multispecific binding by antibodies from normally heterogenous responses. The work was based on the expectation that, for the most part, significant degrees of inhibition would occur only when clonally restricted subpopulations of antibody-secreting cells were being observed. This situation was realized by employing in the plaque assays red cell-bound test haptens bearing features both similar and dissimilar to the immunizing hapten. Various alternate haptenic structures, synthesized in this laboratory, were used. Studies were carried out with spleen cells from rabbits immunized against one of the large haptens. Inhibition curves (PFC counts vs log conc. of inhibitor) and other inhibition data routinely showed PFC subpopulations whose antibodies had high affinity for (1) the immunizing hapten, (2) the alternate assay hapten, and (3) one or more unrelated substances (from a panel of 20). The inhibition curves were frequently steep in slope indicating that a restricted population of antibodies was being observed. Thus, it appears that multispecificity can be demonstrated with limited testing, is functional in hapten-directed complement-dependent hemolysis, and is a property of at least some components of normally raised antibodies.

A more general approach to studying multispecificity is underway in this laboratory. Direct binding to disparate structures will be demonstrated by screening radioactively labeled, specifically isolated antibodies with small affinity chromatography columns. Each of many columns will contain an adsorbent with a different substance bound to it. Binding will be demonstrated by counting the columns after passage of the labeled antibody mixture plus wash, and/or by counting the specifically eluted antibody following the passage and wash. Last year the assay was shown to be capable of measuring specific binding of as little as 0.1% of the total antibody.

During the past year, effort was directed toward synthesis of large, potential haptens for the screening columns. Many of these compounds are to be pentapeptides with various N-terminal structures and varying sequences in the N-terminal three residues. The C-terminal portions will bear functional groups that can be coupled readily with adsorbents or other carriers. In the interest of economizing effort, a number of strategies and techniques of peptide synthesis were either selected or developed de novo. Currently, a number of di and tripeptide fragments are being made in considerable quantities (e. g., 5 to 20 grams). These peptides have terminal groupings which allow racemization-free couplings and are purified by crystallization whenever possible. Coupling methods were selected to avoid the necessity (with few exceptions) of using side-chain protecting groups. Some innovations for peptide synthesis arising from this work during the last year are: (1) methods of synthesis and use of t-Boc-hydrazides of N-CBZ serine, threonine, asparagine and glutamine. These useful intermediates can serve as either C- or N-terminal components in synthesis of optically active peptide fragments, (2) methods of preparing active esters and hydrazides from alcoholic solutions. The products often crystallize out of the reaction mixtures, (3) standardized cold azide coupling procedures with use of mild bases.

Further improvements were made in the active ester approach to radioiodination through amino groups (on proteins, polymers, antibodies, etc). It was feasible to start the procedure by incorporating radioiodine into a very large molar excess of phenolic active ester (Bolton-Hunter compound). In addition to increased efficiency, mainly the monoiodo active ester was produced (rather than the somewhat bulkier diiodo label). The excess unlabeled compound and any diiodo form was shown to be separable by chromatography on silica gel)TLC or columns). A large number of solvent systems for this separation were investigated. Unlabeled mono and diiodo active esters serving as reference materials were synthesized by alternate routes by methods developed during the course of this work.

Eventual characterization and study of multispecific antibodies will involve amino acid sequencing within light and heavy chain variable regions. In the interest of reducing the labor of such work, new improvements in sequencing methodology have been explored as ideas have arisen. It was mentioned in last year's report that it was possible to convert the immediate products of an Edman degradation i.e., the thiazolinones to phenylthiocarbamyl amino acid alkylamides for susequent identification and quantitation. In collaboration with Dr. Ettore Appella (NCI) this approach has been systematically explored in recent months in this laboratory. The method has been sucessfully adapted to manual and automated solid-phase sequencing. Considerable advantage is gained over the conventional PTH conversion method in the time saved, he quality of separations, and the quantitation, especially in regard to certain polar amino acids. A report of this work will be given at the forthcoming First International Conference on Solid-Phase Methods in Protein Sequence Analysis (Boston, May 21-23, 1975).

No previous attempt has ever been made to systematically explore disparate multispecificity of antibodies. This phenomenon may play an important part in providing breadth to specific immune competence and adaptibility for immunity to new pathogens. Multispecificity could conceivably be involved in autoimmune mechanisms. Work in this area will proceed with the technical aspects of the binding assay and screening system, then with measurements of natural antibody levels in non-immune IgG and the actual screening of specifically isolated antibody.

Project Subtitle II: Synthesis and/or Chemical Modification of Antigens: Studies of Specific Immune Responses at the Cellular Level

The use of haptenated Ficoll derivatives prepared by methods developed in this laboratory and now described in the literature (see Publications) has expanded to a number of laboratories within and outside of the NIH. This versatile type of synthetic, thymus-independent antigen is finding a number of applications in studies of B cell activation. Collaborative work involving Ficoll derivatives is continuing with Drs. Paul, Zitron and Mosier in the Laboratory of Immunology. Major results will be summarized in their respective annual reports.

The derivatization reactions used for Ficoll appear to be widely applicable to many polysaccharides including bacterial cell wall components levan, dextrans inulin and agarose. Hydrazide groups have been introduced into Ficoll which can be converted to reactive acyl azides groups allowing Ficoll to be covalently bound to proteins or cell walls. Such a strategy may be useful in certain studies involving Ficoll antigens.

During the past year I have collaborated with Drs. Hillel Koren and John R. Wunderlich (NCI) in Dr. Koren's study of in vitro induction of hapten-specific, cytotoxic T cells. Sensitizing and target cells were conjugated with tripeptide derivatives terminating in NIP or arsonophenylazo groups. In syngeneic systems (C57BL/6 mice) it has been possible to stimulate cytotoxic cells from their noncytotoxic T cell precursors. The latter can exist as memory cells produced by prior i.p. injection of hapten-modified spleen cells into syngeneic donors. Specificity in these systems has been shown by the following observations: (1) the presence of sensitizing hapten on the target cells is necessary for optimal cytotoxicity; (2) inhibition studies with nonradiolabedled cells show that only cells modified with the sensitizing hapten will block cytolysis; (3) soluble macromolecular conjugates of one hapten inhibited cytolysis by cells sensitized with that hapten but not by cells sensitized with another somewhat different hapten.

The above studies provide a model system for studying the nature and extent of specificity exhibited by cytotoxic T cells. Information of this kind can aid in elucidating mechanisms of specific cellular immunity which in turn may lead to ways of controlling cellular immunity as it affects the development of neoplastic or autoimmune diseases. Studies of in vitro cytotoxicity are to be extended with the use of additional large haptenic compounds such as those being synthesized for studies described under Project Subtitle I.

Publications:

Inman, J.K.: Multispecificity of the Antibody Combining Region and Antibody Diversity. In: The Immune System: Genes, Receptors, Signals. Sercarz, E.E., Williamson, A.R., Fox, C.F. (Eds). New York, N.Y., Academic Press, Inc., 1974, pp. 37-52.

Inman, J.K.: Covalent Linkage of Functional Groups, Ligands and Proteins to Polyacrylamide Beads. In: Methods in Enzymology Vol. 34, Affinity Techniques, Enzyme Purification: Part B. Jacoby, W.B. and Wilchek, M. (Eds.). New York, N.Y., Academin Press, Inc. 1974, pp. 30-58.

Inman, J.K.: Thymus-independent antigens: The preparation of Covalent, Hapten-Ficoll Conjugates. J. Immunol. 114, 704-709, 1975.

Koren, H.S., Wunderlich, J.R. and Inman, J.K.: In vitro Development of Cytotoxic T-Cells Specific for Hapten Associated Antigens on Syngeneic cells. Transplantation Proc., In press, 1975.

Project No. Z01 AI 00036-10 LI
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 - June 30, 1975

Project Title: Immunoglobulin Genetics: Regulation of Gene Expression and Lymphoid Differentiation.

Previous Serial Number: 146

Principal Investigator: Rose G. Mage, Ph.D.

Other Investigators:
Glendowlyn O. Young-Cooper, M.S., LI/NIAID
Aftab A. Ansari, Ph.D., LI/NIAID
Andrew J. Drexler, M.D., LI/NIAID
Theodore Werblin, M.D., LI/NIAID

Cooperating Units:
Dr. Ettore Appella, LCB/NCI
Drs. S. and R. Tosi, Rome, Italy
Dr. A. Chersi, Rome, Italy
Dr. M. Carta, Rome, Italy
Dr. S. Dray, Chicago, Ill.

In addition, this year we tested samples for, or supplied reagents or allotype defined rabbits to the following investigators: Dr. R. Asofsky, LMI/NIAID, Dr. R. Cathou, Boston, MA., Dr. L. Claflin, Ann Arbor, MI., Dr. M. Feldmann, London, England, Dr. M. Frank, LCI/NIAID, Dr. F. Finkelman, LI/NIAID, Dr. I. Green, LI/NIAID, Dr. R.R. Fox, Bar Harbor, ME., Dr. R. Hamers, Brussels, Belgium, Dr. C. Janeway, LI/NIAID, Dr. K. Knight, Chicago, Ill., Dr. M. Koshland, Berkeley, CA., Dr. M. Margolies, Boston, MA., Dr. P. Montgomery, Phila., PA., Dr. W.E. Paul, LI/NIAID, Dr. O. Roholt, Buffalo, NY, Dr. D. Sachs, Immunol. Bran.-NCI, Dr. A. Sandberg, LMI/NIDR, Dr. M. Waxdal, LI/NIAID, Dr. B. Wolf, Phila., PA.

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	5-1/2	1		6-1/2
Professional	3-1/2			3-1/2
Other	2	1		3

Project Description:

Keyword Descriptors: Immunoglobulins, genetics, rabbits, allotypes, regulation, gene expression, lymphoid differentiation, immune responses, antigenic determinants, protein structure

The objectives of this study are:

To define by combined immunological and chemical approaches, the chemical nature of structural differences between genetically controlled polymorphic forms of immunoglobulins and between different classes, subclasses, types and subtypes of their polypeptide chains. To utilize this information about the location and chemical nature of the markers on various chains in performing and interpreting breeding studies. To utilize defined reagents for various chains and markers in affecting phenotypic expression of alleles or chain types, studies of membrane-associated immunoglobulins and cell transfer studies. The overall objective is to understand the mechanisms which lead to the synthesis of highly specific antibody molecules, the nature of the genes which influence and control immune responses, and the regulation of gene expression during lymphoid cell differentiation.

Rabbits of known allotype are bred: to produce homozygous rabbits of all combinations at four or more genetic loci; to produce heterozygotes for use in specific experiments and as F1 progeny in linkage studies; to produce F2 and backcross progeny in linkage studies; to produce allotype-suppressed rabbits; and to study the genetics of immune responses to a variety of antigens and vaccines.

Sera of rabbits from the above categories are collected and allotyped by gel diffusion and hemagglutination techniques. The sera are used as sources of IgG and light and heavy polypeptide chains for chemical sequence studies and for immunizations of rabbits and other animals.

Rabbits of defined allotype are immunized with IgG, light chains or other antigens such as bacterial vaccines, hapten-protein conjugates and (for idiotype studies) purified antibodies or IgG fractions and subfractions. Antisera are tested by qualitative and quantitative immunologic and electrophoretic techniques.

Allotypes, types, subtypes and idiotypes are quantitated by: radial immunodiffusion; hemagglutination inhibition; quantitative precipitation of iodine labeled proteins with specific antisera; binding of labeled proteins to immunoabsorbents; inhibition of binding of the labeled antigens to immunoabsorbents. Immunoabsorbents are also prepared and used to purify antibodies, antigens and peptides of known specificity.

Cells from newborn and developing normal and allotype suppressed homozygous and heterozygous rabbits are labeled with iodinated or fluorescein-conjugated anti-allotype reagents. They are also cultured *in vitro* and assayed for immune responses after exposure to antigens or proliferative responses after exposure to mitogens or anti-allotype reagents.

Purified light and heavy chains or fragments of these are obtained from IgG of normal sera or specifically purified antibodies of known allotype as well as from sera of allotype-suppressed rabbits. These are studied using a variety of methods for sequence determination including automated sequencing.

In order to carry out all the studies, we breed and maintain large numbers of rabbits in our own colonies. We produce and characterize large volumes of antisera including anti-allotype antisera made with a variety of donor and recipient combinations, anti-idiotype antisera, antibodies of restricted heterogeneity, and other anti-immunoglobulin reagents. We purify normal and antibody immunoglobulins from large volumes of rabbit sera for use as immunizing antigens, for structural studies, and for other studies described below.

A homologue of human β_2-microglobulin, the light polypeptide chain of HL-A antigens (major human transplantation antigens) has been purified from the urine of rabbits with renal tubular damage. A sensitive assay to detect the protein has been developed. Disc electrophoresis experiments have been conducted which have led to a purification and characterization of the molecule. We are currently determining its amino acid composition and preparing enough purified material from individual rabbits for amino acid sequencing. We hope to discover polymorphism which has not been found in man and which will permit genetic and linkage studies to be conducted with our rabbits. We also plan to prepare antisera which will be useful for the genetic studies as well as our studies of lymphoid cell differentiation.

RML

Peptides which retain antigenic determinants characteristic of the V_H (a3) and kappa chain (b4 and b5) allotypes have been specifically purified by affinity chromatography on immunoabsorbents with purified anti-allotype antibodies bound to them. Radioimmunoassays have been developed to measure the antigenic activity of the peptides. The abilities of a series of a3 preparations to inhibit the binding of labeled purified anti-a3 antibodies to a3-IgG-Sepharose were quantitated on a molar basis. The order was IgG> heavy chain>tryptic digest of heavy chain>citraconylated heavy chain>tryptic digest of citraconylated heavy chain>immunoabsorbent-purified tryptic peptide$\simeq$immunoabsorbent-purified thermolysin peptide. Considerable loss of conformation appears to occur as one goes from the native molecule to the peptide. In contrast an immunoabsorbent-purified peptide from b4 light chain has a considerable amount of retained conformation as judged by inhibition of binding of labeled light chain to anti-b4-Sepharose.

We produced antibody to the A12 allotype for the first time and are using this reagent along with other reagents previously prepared in our continuing tests for recombination within the heavy chain linkage group of our rabbits. Tests conducted by members of Dr. Dray's group in Chicago, on sera of the family of rabbits in which an apparent recombination did occur have shown that the V_H marker remained in its linked relationship to a μ chain marker when the recombination occurred. The markers on both parental chromosomes for the α chain were indistinguishable and were thus uniformative. The linked expression of V_H amd C_γ markers has been documented in normal and allotype suppressed rabbits using recently developed methods for quantitating the C_γ markers A14 and A15 in whole sera. Antisera to C_γ markers (A14,A15 and A11) have been found ineffective in establishing chronic allotype suppression possibly because receptors with these determinants are absent or inaccessible to antibody on the surface of the cells which will differentiate to produce these IgG molecules. We have pointed out that this observation as well as a number of other differences distinguish the allotype-suppression phenomenon in SJL x BALB/c mice.

Systematic examination of the cross-reactions of anti-b-locus allotype alloantisera both qualitatively and quantitatively has led to the construction of a "map" of the serological relationships between b4, b5, b6 and b9. The main features of this "map" are minimal and symmetrical distances between b5 and b6 and maximal distances between b4 and b9. The other distances lie between these two extremes. Since the b6 allotype has the most in common with every other allotype and b9 the least, the b6 antigen and anti-b6 are most frequently involved in cross-reactions whereas b9 and anti-b9 are involved the least. This information leads to the conclusion that for the most specific anti-b-locus allotype antibodies, the recipients of choice are heterozygous b^5b^6 for anti-b4 and anti-b9, b^4b^5 for anti-b6 and b^4b^6 for anti-b5.

Lymphocyte surface markers and functional responses appear asynchronously in the spleen cells of developing rabbits. From birth through the first month of life, about 15% of the cells had detectable surface immunoglobulin while about 45% had C3 receptors. In adults as many as 77% of spleen cells had easily detectable surface Ig but the proportion of C3 receptors remained about 45%. The proliferative response to anti-allotype antisera was present at birth and at adult levels by one month of age. The proliferative response to pokeweed mitogen was low during the first week but comparable to the adult response by two weeks of age. *In vitro* responsiveness to concanavalin A was present at low levels at birth and increased sharply during the first week. We did not observe significant stimulation of spleen cells from neonatal to four week old rabbits by lipopolysaccharide from *Salmonella typhosa*.

Sensitive and rapid methods to detect allotypes and anti-allotype antibodies by solid phase radioimmunoassays were developed. Disappearance of maternal allotype in the homozygous offspring of heterozygous mothers was quantitated and fell below the limits of detection (0.5 μg/ml) by twelve weeks of age. To test if this potential tolerogen affects the immune response, immunizations of young animals with Ig of maternal allotype are being conducted. Immunization after 8 weeks of age results in what appears to be a normal anti-allotype response. Immunization at four weeks of age results in a delayed onset of a normal appearing response. At two weeks (so far, in a limited number of animals) no response was observed.

A report of Strosberg and coworkers that a rabbit with pherotype a1a2a3, b4b5b6 was observed upon immunization with a vaccine of *Micrococcus lysodeikticus* led us to attempt to repeat this important observation. The first 12 animals we studied did not produce allotypes other than those already expressed (and expected from their known genotypes) although all produced large amounts of antibodies of partially restricted heterogeneity. Additional animals are currently under immunization.

It is becoming increasingly apparent that both the chemistry and the genetics of immunoglobulins are analogous in many respects to the cellular isoantigens involved in transplantation, cell-cell recognition systems, tumor antigens and other cell surface markers. Our studies thus help to define the genetic mechanisms which lead to the synthesis of highly specific antibody molecules and the nature of the genes which influence and control immune responses. The genetic markers are valuable for the study of cell surface receptors and the role they play in differentiation of immunocompetent cells. Our studies help delineate the chemical basis for the specificity of these protein antigens and antibodies and the nature of structural and regulatory genes in complex mammalian genetic systems. The breeding and cellular studies which can be done with rabbits provide information applicable to the homologous human transplantation and immunoglobulin systems. An understanding of the chemistry and genetics of these antigens, the factors which control their genetic expression, and their biological roles is thus of broad fundamental interest.

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We are continuing most of the studies described. New areas of emphasis include our studies of the structure and genetics of rabbit β_2-microglobulin, development of conditions for in vitro culture of rabbit cells for studies of mechanisms of allotype suppression, and a recently initiated collaboration with Dr. L. Claflin on the site specificity of rabbit antibodies to phosphoryl choline and the influence of a and b locus allotypes on the specificity.

Appendix - Contract Number: N01-AI-81406 Maintenance and Breeding of Rabbits of Known Genotype and Their Use for Immunological Studies

The objectives and methods are as outlined above. Specifically the contract facility performed over 1400 injections, 5000 bleedings, as well as other miscellaneous technical procedures. Various short-term projects and 30 long-term experiments involving breeding, immunization and suppression were conducted. In addition to providing allotype-defined antigens and anti-allotype reagents, and conducting routine breeding, the following special experiments were conducted: breeding to detect recombination within the heavy chain linkage group; breeding of C6 deficient rabbits (collaboration with Dr. M. Frank, LCI/NIAID); breeding and immunization of young animals to evaluate tolerogenic effects of maternal allotypes (collaboration with Dr. T. Werblin); production of antisera to five different purified pokeweed mitogen fractions (Dr. M. Waxdal); production of large volumes of antisera to mouse $IgG2_a$, $IgG2_b$, IgG1, and kappa chains (collaboration with Dr. R. Asofsky, LMI/NIAID and Dr. W.E. Paul, LI); production of anti-hapten antibodies (Dr. E.B. Merchant and Dr. J.K. Inman, LI); hyperimmunization of groups of rabbits with vaccines of type III pneumococci, M. lysodeikticus, and capsulated pneumocci (R_6 strain). Large numbers of young rabbits were bled for Drs. M. Rand and M. Potter (LCBGY/NCI) so that they could screen sera for complement activity and low cytotoxicity. Appropriate animals were then given to them. Similarly young rabbits were bled for Dr. A. Sandberg (LMI/NIDR) so that sera useful as a source of complement in in vitro studies of bone resorption could be selected. Weekly bleedings from selected animals were then supplied for several months. The contract facility housed and fed an average daily inventory of 500 rabbits, carried on a weekly breeding program, weaned and tattooed approximately 500 young rabbits, and maintained records on all the above-mentioned activities. Most of the reagents and rabbits made available to the other investigators listed on the first page of this report were produced and supplied through this contract.

Honors and Awards:

Allergy and Immunology Study Section, Member Federation Proceedings Editorial Board, Advisory Editor Immunochemistry; Associate Editor J. Immunol.; American Association of Immunologists - Committee on status of women and minorities; Invited workshop co-chairman - Depression of Ig synthesis by anti-Ig sera-2nd International Congress Immunol., Brighton, England, Invited participant - workshop on "Markers of Rabbit Cells and Other Aspects of Immunology in Rabbits" to be held during European Immunology Meetings, Sept. 1975; Invited Contributor to Vol. 27 Transplantation Reviews for articles in area of "Antibody Suppression of Gene Products"; Invited compiler of a tabulation of "Immunoglobulin Allotypes" for CRC Handbook of Biochemistry and Molecular Biology 3rd Edition (Dr. G. Fasman, Ed.); Instructor - "Immunogeneitcs" tutorial seminar.

PUBLICATIONS:

Freedman, M.H., Pincus, J.H., Yeger, H., McKenney, J.A., and Mage, R.G.: Studies of hyperimmune restricted and partially restricted anti-pneumococcal polysaccharide antibodies from allotype-defined pedigreed rabbits. I. Preparative liquid isoelectric focusing of the antibodies and characterization of the isolated fractions by electrophoretic techniques. Eur. J. Immunol. 4: 553-560, 1974.

Mage, R.G., Pincus, J.H., Alexander, C., and Freedman, M.H.: Studies of hyperimmune restricted and partially restricted anti-pneumococcal polysaccharide antibodies from allotype-defined pedigreed rabbits. II. Allotypes and idiotypes in sera and electrofocused fractions. Eur. J. Immunol. 4:560-564, 1974.

Elfenbein, G.J., Harrison, M.R., and Mage, R.G.: Appearance of lymphocyte surface markers and functional responses in neonatal and young rabbit spleens. Cellular Immunol. 15: 303-311, 1975.

Appella, E., Chersi, A., Rejnek, J., Reisfeld, R. and Mage, R.: Rabbit immunoglobulin lambda chains: Isolation and amino acid sequence of cysteine-containing peptides. Immunochem. 11: 395-402, 1974.

Mage, R.G.: Remarks on allotype suppression in rabbits. In Katz and Benacerraf (Eds.): Immunological Tolerance: Mechanisms and Potential Therapeutic Applications, New York, Academic Press, 1974, pp. 531-536.

Mage, R.G.: Depression of immunoglobulin synthesis by anti-immunoglobulin sera. In Brent and Holborow (Eds.): Progress in Immunology II: New York, American Elsevier, 1974, pp. 379-380.

Mage, R.G.: Normal and altered phenotypic expression of immunoglobulin genes. Fed. Proc. 34: 40-46, 1975.

Landucci-Tosi, S., Mage, R., Young-Cooper, G.O. and Tosi, R.M.: Serological relationships between rabbit immunoglobulin allotypes controlled by the b locus. Immunochem. In press.

Mage, R.G.: Immunoglobulin Allotypes. In Fasman, G.D. (Eds.) CRC Handbook of Biochemistry and Molecular Biology (Third Edition) Cleveland, Ohio, CRC Press, in press.

Project No.: Z01 AI 00037-08 LI
1. Laboratory of Immunology
2. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Immunogenetics of Mouse Immunoglobulin and Genetic Control of Antibody Responses.

Previous Serial No.: 147 and 148

Principal Investigator: Rose Lieberman, M.A.

Other Investigators:

Project Subtitle I:	Dr. M. Potter, LCBGY/NCI Dr. M. Vrana, LCBGY/NCI Mr. W. Humphrey, Jr., LI/NIAID
Project Subtitle II:	Dr. M. Potter, LCBGY/NCI Mr. W. Humphrey, Jr., LI/NIAID
Project Subtitle III:	Dr. M. Potter, LCBGY/NCI Dr. W. Paul, LI/NIAID Mr. W. Humphrey, Jr., LI/NIAID

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	2			2
Professional	1			1
Other	1			1

Project Description:

Project Descriptors: Genetics of mouse immunoglobulins; genetic control of immune response.

Project Subtitle I: Study of Immunoglobulin Determinants in Mice With Homologous Antisera to Determine Genetic Control of the Structure of Immunoglobulin Molecules.

1. To prepare homologous anti-allotypic and idiotypic (myeloma-specific) antisera highly specific for determinants on various parts of the different chains of mouse immunoglobulin molecules.

2. To study genetic control of immunoglobulin determinants.

3. To use genetically controlled immunoglobulin markers in studies of antibody synthesis.

4. To determine if identical molecules, based on biologic activity and identification of Fab determinants, can be found in myeloma proteins from independently induced plasma cell tumors.

Methods:

1. Preparation of immunoglobulin immunogens (Fc, Fab and light chains) from normal and plasma cell tumor bearing inbred mice.

2. Immunization of inbred strains of mice with specific normal or myeloma immunoglobulin molecules or fragments of immunoglobulin molecules (chains).

3. Identification of immunoglobulin determinants with homologous antisera using immunoelectrophoresis, Oudin and Ouchterlony gel diffusion methods, radioisotope tagging, precipitin inhibition method, hemagglutination inhibition techniques and immunoabsorbents.

4. Selective breeding of mice to determine linkage, allelism, independent assortment and crossover of genes. Production of coisogenic strains to propagate and isolate genetic markers in a known genetic environment. Chromosome mapping of genetic markers of immunoglobulin determinants with other genetic markers as coat color, etc., to facilitate easy identification of immunoglobulin genetic markers and also to determine the location on the chromosomes of genes controlling specific immunoglobulin determinants.

5. Development of techniques employing immunoglobulin determinants as genetic markers in problems of transplantation, histocompatibility, transfusion reactions, immunization and antibody synthesis.

Heavy chain constant region (C_H) determinants have been identified on γ_1, γ_2a, γ_2b and γA myeloma proteins of BALB/c origin and a γA and γ_2b myeloma protein of C57BL origin (CB20). No determinants have been identified on γM or γG3 myeloma proteins. All the C_H antigenic determinants are controlled by closely linked genes. Twelve heavy chain antigenic groups have been identified; nine in inbred strains and three in wild mice.

In addition to C_H antigenic specificities, antigenic determinants present in other regions of the molecule, namely, the Fab or heavy chain variable (V_H) and possibly the light chain constant (C_H) regions have also been identified. Homologous antisera prepared with specific myeloma proteins usually only identify the idiotypic determinants present on the Fab region of that myeloma protein used to prepare the antiserum. Occasionally cross reactions between idiotypes present on different myeloma proteins have been observed. This has been true of several different IgA myeloma proteins that bind phosphorylcholine. Other myeloma proteins have been found with similar antibody specificities. Many myeloma proteins have been found to have anti-levan activity. Homologous antisera are presently being prepared to the idiotypic determinants of these proteins. One group of 11 proteins bound B2→1 fructosans, a second group of 2 proteins bound fructosans probably of B2→6 linkage. Anti-idiotypic sera prepared to 10 of the proteins identified multiple idiotypic determinants. Each protein used for immunization had its own unique

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individual idiotypic specificity (IdI) and in addition most of the proteins carried 2-9 cross-specific or ahared idiotypes (IdX) that were found only among the 13 levan binding myeloma proteins and were not present in 106 other myeloma proteins of various classes that did not bind levan. Most of the IdX determinants and four IdI determinants of the B2→1 fructosans binding group were located in the antigen binding site. The multiplicity of antigenic differences in this functionally related group of immunoglobulins reveals a heterogeneity in the V-regions that appears to be unrelated to the binding.

It is difficult to study the genetic control of the diversity of antibody using normal antibodies since these are mixtures of many types of molecules. Myeloma proteins are pure molecular forms of immunoglobulins and these can be obtained by experimentally inducing plasma cell tumors in some strains of genetically defined inbred mice. In addition, many of these proteins have been found to have antibody activity. Immunoglobulin determinants on both the common and variable regions of the heavy chains as well as conformational determinants involved in the antibody site provide tools to study the genetic control of the variability of antibodies. The finding of 5 identical myeloma proteins appears to indicate repetition of genetic mechanisms that control diversity of antibody production. The finding of multiple V region markers associated with specific anti-hapten binding sites (anti-fructosans) enables us to do studies to determine the genetic control of these V region markers and to correlate the serologic findings with the structional data. Many of these idiotypic (anti-levan) markers have been found in normal and immune sera of mice immunized with levan and show a definite distribution pattern among the inbred strains the relationship of the genes controlling these anti-hapten idiotypic determinants to other immunoglobulin structional genes is presently being investigated.

Project Subtitle II: Genetics of V-regions.

Identify and map as many genes controlling immunoglobulin variable regions (IgV genes) as possible in order to determine the frequency of recombination between various structural genes for anti-binding sites of immunoglobulin and between these genes and genes controlling constant region structures.

Fab idiotypic determinants present on myeloma proteins of BALB/c origin are serologically identified by homologous antisera. Among the myeloma proteins that have been determined to have antibody activity are the 13 levan binding myeloma proteins, 11 of which bind B2→1 linked fructosans and 2 of which bind B2→6 linked fructosans. Some of the multiple shared idiotypic determinants present on the myeloma proteins with 11 B2→1 linked fructosans activity have been found to be present in normal and immune sera of BALB/c, and other strains.

A survey of strains for some of the idiotypic V-region markers associated with the B2→1 linked fructosans site show that these are present also in A and AL strains and in the Bailey recombinant CxBJ and are absent in CBA, C3H, B6 and B10 inbred strains and in CB20 congenic strains and in the recombinant CxBI strains.

Mapping of the genes controlling the anti levan idiotype in relation to other structural V-region markers (phosphorylcholine, aso arsonate, streptococcusal, γ1-3 derivition NIP, NBR, etc.) will be done.

The identification of genes controlling immunoglobulin variable region determinants permits studies of mechanisms of antibody diversity and formation of antibody molecules.

Project Subtitle III: Genetic Control of Antibody Responses to Myeloma Proteins of Mice.

It has been shown in the mouse that the immune response to a BALB/c $\gamma 2a^{\kappa}$ type myeloma protein (MOPC173) is controlled by the immune response gene Ir-IgG. In mice with IgC$_H$ allotypes different from BALB/c, the Ir-IgG gene was found to be linked to H-2 types b,bc, p, r, s and v not to a, d, k and q. The response of mice of the H-2^b type and lack of response of H-2^a mice permitted us the opportunity to map the Ir-IgG gene in the H-2 region by investigating the immune response of the H-2^a/H-2^b recombinants that were available. These recombinants included B10.A (1R), B10.A (2R), B10.A (3R). B10.A (4R), and B10.A (5R). It was found the 3R, 5R and 4R gave a high response while 1R and 2R did not. On the basis of prior mapping of other Ir genes, e.g. Ir-I and Ir-IgA, it was shown that Ir-IgG was located between the Ir-IgA, Ir-I complex and Ss-Slp. In that study we postulated that the allotype present on the C$_H$ (Fc) region was recognized by specific thymus-derived (T) lymphocytes and that an immune response to the idiotype present on the Fab could only be elicited when the allotypes of the immunized mouse differed from BALB/c. On these premises, irrespective of the differences in the V$_H$ regions (idiotypes), the same H-2 linked Ir gene should be involved in responses to all myeloma proteins of the same class. To test this view, the immune response to six different BALB/c γ2a myeloma proteins was investigated in inbred, H-2 congenic and recombinant strains.

The immune response to six different myeloma proteins of BALB/c origin was determined. Four of the γ2a myeloma proteins were of the kappa type (MOPC173, LPCI, UPC10 and RPC5); two were of the lambda type (HOPC1 and S5444). All the six myeloma proteins have the same C$_H$ region allotype determinants ($G^{1,6,7,8}$). Each of the four kappa type proteins has its own unique idiotypic specificity which does not cross-react with that of any of the others. The two lambda types in addition to possessing unique idiotypic specificities, also have cross-reacting idiotypic determinants.

Among the inbred strain of various H-2 types and Ig allotypes the immune response for the six γ2a myeloma proteins was remarkably consistent. No antibody response was observed in mice with the same allotype as BALB/c, including one strain of the H-2^b type. In strains of mice with allotypes different from BALB/c, a high immune response (mean log 2 HI titer 8 - > 12) was found in H-2 types b, r, s and v for 2 or 3 of the k types and for 1 or both of the lambda types. A low or absent immune response was found in H-2 types a, d, k and q for at least 2 of the 4 kappa types and for one of the lambda types. One notable exception was the high response of RF (H-2^k) to

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UPC 10, a kappa type γ2a protein. Among the kappa types, a high response was found in $H\text{-}2^b$ (B10) and a low response in $H\text{-}2^a$ (B10.A). Among the $H\text{-}2^a/H\text{-}2^b$ recombinants, 1R and 2R gave a low response while 4R and 5R gave a high response. The response of 4R while present, was always less than that of 5R or B10. The data indicate that regardless of the V_H region of the myeloma protein, the same Ir gene (Ir-IgG) is employed in the immune response to κ type γ2a BALB/c myeloma proteins.

The findings with the λ type γ2a proteins were quite different. Firstly, the results on repeated experiments revealed a marked variability among individuals within a strain, a finding not observed with the κ type proteins. For S5444 all the B10 mice gave a high response whereas for HOPC1 only 3/22 gave a high response in three separate experiments using different preparations of HOPC1. Both the S5444 and HOPC1 gave low responses in the recombinants 2R, 4R and 5R. However, there were some inconsistencies in these latter groups. Among the 4R, 2/18 gave a good response and among the 5R, 2/23 gave a good response to HOPC1. Despite this variability, it was still clear that the immune response to HOPC1 was linked to the H-2 complex. Thus, all individuals of the B10.M strain gave a very high immune response (log 2 H-1 mean titer > 12).

The data support the concept that the genetic control of responsiveness to γG (γ2a) κ myeloma proteins involves recognition of constant region all-type associated determinants. This most likely involves antigen recognition by T lymphocytes, but our data do not establish such a role for T lymphocytes. However, one strain (RF) responded to UPC10, but not MOPC173 or LPC1, suggesting in this case a recognition limited to a unique antigen of UPC10.

The situation of the lambda γ2a proteins is much less clear, although it seems possible that an Ir gene distinct from, although linked to, that controlling responsiveness to κ γ2a proteins may be involved.

Histocompatibility-linked Ir genes play an important role in antigen-recognition by T lymphocytes and in the control of susceptibility and resistance to a variety of diseases. The mechanisms by which these critical functions are mediated is still unknown; their delineation should be important in specific manipulation of the immune response. In addition, Ir-genes also serve as genetic markers to study gene products at the cellular level.

Honors and Awards:

Visiting Professor, University of Miami School of Medicine, Department of Microbiology, November, 1974 to May, 1975.

Advisory Editor - Immunochemistry

Program Committee - American Association of Immunologists

Visiting Scientist at Weizmann Institute - September, 1974.

Publications:

Lieberman, R., Potter, M., Humphrey, W., Jr., Mushinski, E.B. and Vrana, M.: Multiple individual and cross-specific idiotypes on 13 levan-binding myeloma proteins of BALB/c mice. *J. Exp. Med.*, in press, 1975.

Lieberman, R., Paul, W.E., Humphrey, W., Jr. and Potter, M.: H-2 linked *I-r* genes controlling immune responses to BALB/c IgG ($\gamma 2a$) myeloma proteins having kappa (κ) or lambda (λ) light chains. *Transplantation Proc. Supplement* March, 1975.

Lieberman, R., Potter, M., Mushinski, E.B., Humphrey, W., Jr., and Rudikoff, G.: Genetics of a new IgV_H (T15 idiotype) marker in the mouse regulating natural antibody to phosphorylcholine. *Immunogenetics* 1: 524, 1975.

Lieberman, R., Potter, M. and Humphrey, W., Jr.: Identification and genetics of a cross-reacting marker associated with IgG2a myeloma proteins and normal serum of BALB/c mice. *Immunogenetics* 1: 529, 1975.

Lieberman, R., Potter, M., Paul, W.E. and Humphrey, W., Jr.: H-2 linked Ir genes controlling immune-response to BALB/c IgG (2a) and IgG (2a) type myeloma proteins. *Immunogenetics* 1: 530, 1975.

Project No. Z01 AI 00038-03 LI
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Structural and Activity Studies on Immunologically Important Cells and Proteins

Previous Serial No. 149

Principal Investigator: Dr. Myron J. Waxdal

Other Investigators: Project Subtitle I: Dr. Sten Nilsson, LI, NIAID
Dr. Steven Siegel, LI, NIAID
Ms. Terri Basham, LI, NIAID
Project Subtitle II: Dr. Herbert Reynolds, LCI, NIAID
Mr. Russell Thompson, LCI, NIAID
Project Subtitle III: Ms. V. Vinton, DCRT
Mr. D. Songco, DCRT
Mr. B. Martino, DCRT

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to	Guest Workers	TOTAL
TOTAL	5	3 6/12		8 6/12
Professional	3	6/12		3 6/12
Other	2	3		5

Keyword Descriptors: Mitogen, cell receptor, B and T cells, mitosis, immunoglobulin, secretory component, computer, automatic data processing

Project Description:

Project Subtitle I: Lectins and their interaction with cells

Certain lectins are polyclonal stimulants (mitogens) for immunocompetent cells and as such provide an advantageous approach to the study of triggering in these cells. Five mitogens (Pa-1 to Pa-5) have previously been isolated from pokeweed and characterized both physicochemically and biologically. Only Pa-1, apparently a multimer, stimulates murine B cells to divide and produce immunoglobulin. Blockage of cell division with vinblastine at various times after Pa-1 stimulation showed that those B cells which differentiate and produce immunoglobulin divide only during the first 24 hours of culture. Other B cells, which do not produce immunoglobulin continue division to 72 or 96 hours. This finding supports the existence of subsets of B cells.

To further understand the mechanism by which mitogens stimulate lymphocytes, the isolation of receptors on B and T cells for several lectins has been initiated. Among these lectins are concanavalin A, Pa-1 to Pa-5, lentil lectin and wheat germ agglutinin. The cells are radiolabeled by several techniques, including metabolic and surface labeling. Preliminary evidence indicate that the major cellular receptors from both murine B and T cells are products of the H-2 histocompatibility locus (H-2D, H-2K, and the I complex). It has not yet been established that the pokeweed mitogens bind to the carbohydrate portion of cell surface glycoproteins, as do other characterized lectins. The receptor(s) from human erythrocytes are being isolated and physico-chemically characterized to determine the actual binding site and to analyze its chemical composition.

Chemical modification of the T cell mitogens Pa-2, Pa-4, concanavilin A (a tetramer) and succinyl-concanavalin A (a dimer) with dimethyl suberimidate produced an insoluble covalently bonded aggregate. All of these aggregated proteins stimulate mitosis in murine T cells. Aggregated Pa-2 and Pa-4 strongly stimulate mitosis in murine B cells and also cause immunoglobulin production. Aggregated succinyl-con A also stimulates B cells but does not cause immunoglobulin production. In contrast, aggregated Con A has no B cell activity. The non-immunoglobulin producing B cells which respond to aggregated succinyl-Con A further support the division of B cells into subsets.

These investigations will be continued to firmly establish the relevant mitogen binding sites on immunocompetent cells and to determine the necessary mitogen-receptor interactions for stimulation of these cells.

Project Subtitle II: Structural Studies of Immunoglobulins

Immunoglobulin A (IgA) was isolated from canine serum and colostrum. Free secretory component (FSC) was isolated from colostrum. These proteins were purified by affinity chromatography. Both serum and colostral IgA were susceptible to reduction with dithiothreitol. Colostral IgA released an additional polypeptide chain, bound secretory component (BSC). BSC was shown to be identical to FSC in electrophoretic migration, isoelectric focusing and molecular weight. FSC showed antigenic determinants not shared with BSC. The amino acid composition and NH_2-terminal sequence (12 residues) of FSC was found to be similar to that reported for the human and cow by other workers.

Canine secretory component contains more methionine residues than that from human or cow. This allows the component to be cleaned by cyanogen bromide into several fragments and provides an advantageous position for current studies on the covalent structure of SC and the site of its attachment to IgA.

Project Subtitle III: Laboratory Automation and Data Processing.

In order to derive maximum benefit from the analytical instruments employed, automatic data acquisition, processing, and display is being used and developed. New automated procedures, instrument control and monitoring are also used and under development. In addition to use by members of this section, the computer monitored chromatography system is used by members of LI and the amino acid analysis facilities have been used by other NIH divisions. The heaviest use of the system by LI and other NIH personnel is in processing of data from scintillation and gamma counters. Punched paper tapes from the counters are used to input raw data into the computer. Several software programs have been written to provide statistical analyses of experiments and to provide graphic displays of the calculated data. For this purpose alone many man-hours are saved each week by each user.

The major drawback to the computer system has been the restriction to a single user at a time. This has forced delays of up to several days in processing some data. A new software monitor and associated hardware have been purchased to provide simultaneous multiple user capabilities. In conjunction with the interfacing of more analytical instruments, this will provide constant data acquisition and processing facilities to members of this section, LI, and other NIH users.

Publications:

Waxdal, M.J.: Characterization and Biological Activites of 5 mitogens from pokeweed. Biochem. 13: 3671 (1974).

Waxdal, M.J. and Basham, T.Y.: The B and T Cell stimulatory activities of multiple mitogens from pokeweed. Nature 251: 163, 1974.

Basham, T.Y. and Waxdal, M.J.: The stimulation of immunoglobulin production in murine spleen cells by the pokeweed mitogens. J. Immunol. 114: 715, 1975.

Waxdal, M.J.: Differential stimulation of murine T and B cell populations by purified mitogens from pokeweed. Immune Recognition A.S. Rosenthal, Ed.: Academic Press (NY) 1975, p. 85.

Thompson, R.E., Reynolds, H.Y. and Waxdal, M.J.: Structural composition of canine secretory component and immunoglobulin A. Biochem. (In press).

Project No. Z01-AI00039-01 LI
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Tumor Immunology in Inbred Guinea Pigs

Principal Investigator: Dr. Ira Green

Other Investigators: Project Subtitle I: Dr. Guido Forni, LI/NIAID
Dr. Ethan Shevach, LI/NIAID
Project Subtitle II: Dr. Ernest Manders, LVD/NIAID
Dr. Wallace Rowe, LVD/NIAID
Dr. Michael Collins, Micro. Associates, Bethesda, Md.
Dr. J.S. Rhim, Micro. Associates Bethesda, Md.

Cooperating Units: Laboratory of Viral Diseases, NIAID
Microbiological Associates, Bethesda, Md.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	2-6/12			2-6/12
Professional	1-6/12			1-6/12
Other	1			1

Project Description:

Keyword Descriptors: Lymphocytes, mononuclear cells, receptors, immune response genes, guinea pigs sarcomas, viruses, Transplantation antigens

Project Subtitle I: Immunological Behavior of Viral Induced and Carcinogen Induced Tumors in Inbred and Random Bred Guinea Pigs

Although guinea pigs have a well studied immunological system, tumors of this species have not been as widely employed for tumor immunological studies as compared to tumors of mice and rats. Considering that cellular immunity in guinea pigs is easily demonstrable *in vivo* as well as by a variety of *in vitro* techniques and that cellular immunity persist for many months in guinea pigs, further studies on tumor immunity in this species would appear to offer promise of unravelling the several mechanisms involved

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in tumor rejection. Therefore we were interested to study, in syngeneic guinea pigs, the immunological behavior of two new tumors induced by the Kirsten strain of Mouse Sarcoma Virus (Ki-MSV) in inbred strain 2 and inbred strain 13 guinea pigs and to compare their antigenic structure with one of the more usual strain 13 methylcholanthrene (MCA) induced tumor.

The Ki-MSV used for the induction of these tumors in an RNA C-type virus particularly interesting because of its high efficiency in transforming many animal and human cells. Both these two Ki-MSV tumors, obtained by Dr. J.S. Rhim (Microb. Assoc., Bethesda, Md.) in inbred strain 2 and strain 13 of guinea pigs were found to be non virus producing and lacked any evodemce of murine-leukemia antigens on the cellular membrane, but did contain rescuable sarcoma virus genome. Therefore the study of the membrane antigenic structure and the possibility to induce resistance in syngeneic animals against these tumors was particularly interesting because of their possible similarity with various "spontaneous" animal and human tumors. After a subcutaneous transmission of 10 x 10^6 living cells of these two Ki-MSV tumors and an MCA tumor, 100% of syngeneic animals died in 25-30 days. Immunization with 10 x 10^6 irradiated strain 13 MCA-tumor cells emulsified in Freund complete adjuvant distributed in the footpads markedly reduced the percentage of takes in syngeneic strain 13 animals challenged with the same tumor 20 days after the immunization. In some cases there was an initial take followed by a complete regression 13-30 days after the challenge. Thirty days after the challenge, only 10% of immunized animals still bore a progressively growing tumor. In contrast the same immunization technique or the excision of initially growing tumors failed to induce any protection in the two Ki-MSV tumors. These data are quite different from the findings recently obtained with several new guinea pig Ki-MSV viral induced tumors. These tumors do produce virus and spontaneous regression of these tumors in syngeneic guinea pigs possibly related to the presence of virus coded antigens on the cell membrane is observed. These results fit in well with the findings obtained by others with Ki-MSV induced tumors in the mouse and rat. Thus in several species immunization with non producer MSV induced tumors were found incapable of protecting against a subsequent challenge. However a cell surface antigen common to mouse, rat and monkey non producer cells transformed by MI-MSV has recently been demonstrated by the highly sensitive immuno-electronmicroscopy technique (T. Aoki, _et_ _al_., NCI). Studies are under way to determine whether an identical antigen can be detected on surface of guinea pig non producer cells. We have also compared the behavior of these Ki-MSV and MCA tumors in different allogeneic combinations. Recent studies of guinea pigs histocompatibility antigens have serologically identified two linked systems of alloantigens. One group of antigens is identified with sera raised by cross immunizing inbred strain 2 and 13. The 2/13 antigens are identifiable in all random bred guinea pigs. These antigens have a molecular weight of 25,000 and are restricted to lymphocytes. A second system of alloantigens consist of a group of closely linked loci with at least four alleles (B/C/D/E/) which have a wide tissue distribution and a molecular weight of 43,000 with an 11,000 dalton component. The 2/13 system therefore appears to resemble the immune response associated (Ia) system of the mouse and B/C/D/E system resembles the K and D antigen system of the mouse. It was therefore of great interest to study the growth patterns of these three non lymphoid tumors which serologically appears B^+, 2 or 13 negative, in recipient animals of different histocompatible type. The strain 13 MCA induced tumor grew in syngeneic animals but was rejected by inbred strain 2 (2^+, B^+) and by 13^+, B^+ random bred guinea pigs. The strain 13 Ki-MSV tumor progressively grew in syngeneic animals and in random bred 13^+, B^+, grew and chronically regressed in inbred strain 2 (2^+, B^+) but was rapidly rejected by random bred 13^+, D^+ animals. The strain 2 Ki-MSV tumor grew progressively equally well in syngeneic strain 2 (2^+, B^+), in allogeneic inbred strain 13 (13^+, B^+) and in random bred 13^+, B^+ but was slowly rejected by random bred 13^+, D^+ animals. Therefore the B antigen seems to play a central role in the rejection or acceptance of allogeneic tumors. Differences at the B alloantigen always induce a tumor rejection along with anti-B antibody production strongly suggesting that the B/C/D/E antigens are one of the major transplantation antigens of guinea pigs. The differences at the 2/13 locus are also correlated with patterns of tumor rejection and a T cell dependent cytotoxicity. That is during the growth and chronic rejection of strain 13 Ki-MSV tumor by strain 2 guinea pigs a marked cytotoxicity was observed in T cell population obtained from the peritoneal cells. Whether the tumor rejections and cytotoxicity observed in the strain 2/13 combinations can be explained directly by differences at 2/13 locus is not clear because 2 and 13 antigens are serologically undetectable on the membrane of these non lymphoid tumors. Therefore, the rejection patterns observed may be related to the presence of tumor specific structures, non antigenic in the syngeneic hosts, but recongized in animals with different genetic make up. More probably the rejection patterns and the cell mediated cytotoxicity observed in 2/13 combinations are related to the presence of serologically undetectable minor histocompatibility antigens on the membrane of MCA and Ki-MSV tumor.

These various possibilities are at present under investigation both *in vivo* and *in vitro* using mixed lymphocyte reaction and cellular dependent cytotoxicity. In addition the parallel use in these studies of three different strain 2 leukemias which are respecitvely B^+ and 2^+, B^+ and 2^-, and 2^+ but with some B antigen determinant missing should lead to a more precise identification of the role of K and D like and Ia-like antigens of the guinea pig in the activation of a T cell dependent cytotoxicity and allogeneic tumor rejections.

Project Subtitle II: Induction of Tumors with Viruses in Inbred Guinea Pigs

These studies have two purposes. First, viral induced tumors widely used for studies of tumor immunology have been induced in inbred rats and mice. In these species the growth patterns and immunological behavior of the tumor have been correlated with the presence of viruses and or virally determined antigens on the surface of the tumor cells. In particular, tumors induced by the Kirstein strain of mouse sarcoma virus (KIMSV) have been found to be non antigenic in syngeneic hosts - if the tumor cells are non virus producing. In contrast - if the tumor cells do produce virus, then the tumors are antigenic in the host of origin. The question of whether this behavior of these tumors of mice and rats were peculiar to the virus or to the hosts could be answered if KIMSV induced tumors were produced in other species.

Second, the guinea pig has a number of histocompatibility linked Ir genes. The study of the function of these Ir genes in lymphocytes would be greatly facilitated if the Ir genes and/or antigens closely associated with them on the cell surface could be isolated and chemically identified. Such studies have already been performed with a spontaneous B cell lymphatic leukemia of the strain 2 guinea pig. It is hoped that viral tumors of the lymphoid system can also be induced - such malignant lymphoid cells, and especially thymus derived cells would be especially useful for the isolation and characterization of membrane antigen coded for by the major histocompatibility complex of the guinea pig. The fact that tumor cells are homogeneous populations of cells may be an especial advantage for purposes of chemically characterizing certain of these cell surface proteins.

Newborn and weanling inbred 2 and strain 13 guinea pigs were injected subcutaneously and intraperitoneally with KIMSV and KIMSV with a xenotrophic coat. A high percentage of these guinea pigs developed sarcomas after a latent period of 6-8 weeks. A total of about 10 tumors have been produced. Some of these sarcomas were serially transplantable into syngeneic guinea pigs and have been used for immunological studies described in Subtitle one of this project. These particular tumors were found not to produce virus. Other tumors (these induced with KIMSV with xenotrophic coat were rejected after transplantation into syngeneic animals and these tumors appear to produce virus. Thus the immunological behavior of these producer and non-producer KIMSV viral induced tumors in inbred guinea pigs with respect to their immunogenicity, resembles the behavior of such tumors in rats and mice.

To date no lymphomas have been observed to arise in guinea pigs infected with KIMSV. Other guinea pigs have been infected with Abelson virus (known to produce B lymphocyte tumors in mice); however no tumors are yet evident in these guinea pigs.

These studies will be continued with the hope that lymphoid tumors of the inbred guinea pig can be obtained. Also, under suitable conditions, certain of these viruses could be used to transform population of immunocompetent lymphocytes _in vitro_. If such transformed cells preserved their immunological function, these would provide very useful tools for further chemical analysis of the functions of the immune system.

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Project No. Z01 AI 00040-01 LI
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Genetic Control of Immunocompetent Cell Interactions

Previous Serial No.: 142

Principal Investigator: Ethan M. Shevach, M.D.

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Project Subtitle IV: Dr. P. Cohen, LI/NIAID
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Cooperating Units: Laboratory of Immunology, NIAID, Inselspital, Bern, Switzerland

Man Years:

	NIAID, Lab. Staff	Other NIH Personnel Assigned to	Guest Workers	TOTAL
Total	3-8/12			3-8/12
Professional	2-8/12			2-8/12
Others	1			1

Project Description

Keyword Descriptions: Immune response gene, histocompatibility antigen, T cell, macrophage

Project Subtitle I: Serologic Characterization of Guinea Pig Histocompatibility Antigens

Although the guinea pig has been used for a wide variety of immunologic studies and in particular for studies of genetic control of the immune response relatively little is known about the serologic characteristics of the major histocompatibility complex (MHC) of this species. Because antisera against certain antigens of the guinea pig MHC have powerful effects

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on certain aspects of immune function, it was imperative for us to further investigate the relationships between the antigens identified by cross-immunization of the two inbred strains and another system of guinea pig H antigens developed by deWeck and coworkers by cross-immunization of outbred guinea pigs. In these studies we have also compared some of the characteristics of the antigens of the guinea pig MHC with the well studied murine and human histocompatibility systems.

In these studies we have prepared sera by cross-immunization of the two inbred strains which reveal antigens called "2" and "13". We have also received antisera from Dr. deWeck's laboratory which allowed us to identify a group of H antigens termed the GPL-A antigens. Antisera to the GPL-A antigens identify a single locus with at least three well defined alleles B, C, D; inbred strain 2 and strain 13 animals both bear the B specificity. By the appropriate immunization and absorption experiments we were able to clearly distinguish the 2/13 antigens from the B, C, D antigens. In addition we have identified at least one additional specificity called E which appears to be an additional allele at the GPL-A locus. Breeding experiments demonstrated that the antigens coded for by the GPL-A locus were linked to antigens coded for by the 2/13 genes.

In addition to these studies which involved primarily the testing of cytotoxic antisera on lymphocyte populations for the presence or absence of a given specificity, we have also performed studies on the tissue distribution of the GPL-A antigens and the 2/13 antigens. The results of these studies demonstrated that the GPL-A antigens have a wide tissue distribution and are present on both lymphoid and non-lymphoid cells while the 2/13 antigens are present only on lymphocytes and macrophages. Thus, it is likely that these two groups of antigens although the products of closely linked genes will have markedly different biological properties.

During the course of these studies we have surveyed a number of outbred guinea pig populations for the presence of both the GPL-A antigens and the 2/13 antigens. We are able to characterize most animals as bearing a given GPL-A or 2/13 specificity; we have not encountered a large number of untypable animals and this may indicate that the MHC of the guinea pig is less polymorphic than the MHC of either man or mouse. One population of outbred guinea pigs that has been of great interest is the population of pigmented outbred animals maintained in a close colony at the NIH. Typing of a large number of these animals demonstrated that most were $13^{+}D^{+}$ and a few were $13^{+}B^{+}$. It should be noted that within the NIH colony of guinea pigs there are a small number of animals that are totally deficient in the 4th component of guinea pig complement. Because of the recent demonstration that certain complement components in both man and mouse are linked to the MHC of these species, we studied the relationship between C4 deficiency and the GPL-A antigens of this guinea pig colony. These studies demonstrated that all C4 deficient guinea pigs were B^{+} while guinea pigs with normal levels of C4 were D^{+}. Breeding studies demonstrated a close linkage of the genes controlling the level of C4 with the B antigen. The biological implications of this genetic linkage have still to be determined.

The objective of future studies on the antigens of the guinea pig MHC will be to prepare specific antisera which have the ability to exert effects on the immune response in vitro. We have recently demonstrated that the antigens identified by anti-2 and anti-13 sera are complex and we have been able to prepare a specific serum to one component of the "13" antigen. It is of great interest that this serum is capable of specificially suppressing the in vitro proliferative response of strain 13 cells to the 2,4-dinitrophenyl derivative of guinea pig albumin (DNP-GPA), but not to the copolymer of L-glutamic acid, L-tyrosine (GT). The responses to both DNP-GPA and GT are controlled by Ir genes linked to the 13 alloantigen. These studies demonstrate that the 13 antigen associated with DNP-GPA is distinct from the 13 antigen associated with GT. The ability to prepare sera with very specific biologic properties should allow us to explore in greater detail the relationships betwen histocompatibility antigens and Ir genes.

Project Subtitle II Biochemical Characterization of Guinea Pig Histocompatibility Antigens

Because of the specific inhibitory effect of alloantisera on T lymphocyte proliferation, we have raised the possibility that the alloantisera may in some manner recognize the products of the Ir genes themselves. Biochemical analysis of the antigens recognized by the alloantisera should enable us to better understand Ir gene product function. Our initial studies were performed by labeling strain 2 and strain 13 lymphoid cells with iodine using the lactoperoxidase technique, followed by extraction with a detergent (Nonidet-p-40), and specific immunoprecipitation with the sera under study. The results of these studies demonstrated that antisera recognizing the B antigen produced 2 peaks when electrophoresed on SDS polyacrylamide gels; one peak represented an antigen with a molecular weight of 45,000 and one represented an antigen with a molecular weight of 12,000. Both anti-2 and anti-13 sera precipitated single peaks with molecular weights of 25,000. These initial studies suggested that by molecular weight criteria that the B antigen is similar to the human LA and Four antigens and to the mouse D and K antigens, and that the 2/13 antigens are similar to the antigens defined by the immune response region of the mouse MHC.

We then proceeded to a further analysis of the physiochemical properties of these antigens by a biosynthetic technique which involves labeling of lymphoid cells with ^{3}H-leucine and ^{3}H-Fucose, followed by extraction with NP-40, specific immunoprecipitation, and analysis of SDS-polyacrylamide gels. Using this technique we have characterized GPL-A antigens B, C, D, E and an additional specificity S which is also present on inbred strain 2 and strain 13 lymphoid cells. All of these antigens proved to be glycoproteins as demonstrated by their ability to bind to a lentil lectin column which specifically binds the sugars mannose and glucose; furthermore, all were two component structures--one with a molecular weight of 40,000, the other, 12,000. In contrast, the antigens identified by anti-2 and anti-13 sera were also glycoproteins, but migrated as two molecules, one with a molecular weight of 33,000 and the other 25,000. Sequential immunoprecipitation studies demonstrated that the GPL-A antigens and the 2/13 antigens were on separate molecules. Comparison of the results of the internal labeling studies with the surface iodination studies demonstrated that the 25,000 MW peak seen in the iodination studies was identical with the low MW peak seen on internal labeling. The failure to identify the 33,000 MW peak on surface iodination is still the subject of investigation. Recently, we have shown that in the absence of reducing agents the 33,000 and 25,000 MW chains appear to be part of a single ∿ 58,000 MW structure.

The ability to rapidly label cell surface antigens with these techniques should allow us to determine the biochemical relationships between Ir genes and histocompatibility antigens. Our goals in these studies are to analyze the tissue distribution, particularly intra-lymphoid distribution, molecular heterogeneity and function of solubilized histocompatibility antigens. In particular, we will attempt to demonstrate the biochemical basis for the association of a given Ir gene with an I-region antigen.

Project Subtitle III: The Effect of Alloantisera on Immune Response Gene Product Function

The in vitro proliferative responses of (2 x 13)F_1 lymphocytes to antigens the response to which is under the control of Ir genes is specifically inhibited by alloantisera directed to the alloantigen linked to the Ir gene under study. Thus, the response of F_1 cells to the 2,4-dinitrophenyl derivative of a copolymer of L-glutamic acid, L-lysine (DNP-GL), an antigen, the response to which is controlled by a 2-linked Ir gene, is inhibited by anti-2 serum and not suppressed by anti-13 serum. In contrast, our earlier studies performed in inbred strain 2 and strain 13 animals demonstrated that the responses both to antigens under unigenic control and to a small number of antigens not known to be under unigenic control were inhibited to the same extent. In the present study we studied the effect of anti-2 and anti-13 sera on the responses of strain 2 and strain 13 cells to a wide variety of foreign protein antigens of diverse chemical composition. We also examined the effect of anti-B serum on T cell proliferation.

These studies demonstrated that anti-2 and anti-13 sera had a marked inhibitory effect on the proliferative response of strain 2 and strain 13 cells irrespective of the antigens used for immunization. Analysis of the results revealed that the responses to one group of antigens were inhibited to the same or to a lesser extent than the response to the mitogen phytohemagglutinin. The responses to a second group of antigens composed for the most part of weak immunogens or antigens which had strcutured similarities to antigens under unigenic control were inhibited to a greater extent than the response to PHA. The responses to antigens under unigenic control were always inhibited to a greater extent than the response to PHA. Anti-B serum also exerted a marked inhibitory effect on the responses of strain 2 and strain 13 cells. However, this effect was generalized rather than specific in that the responses to all antigens tested were inhibited to the same extent as the PHA response.

We have concluded from these studies that alloantisera exert multiple effects on the proliferative response of sensitized T cells. They can exert a specific inhibitory effect in the response of (2 x 13)F_1 T cells to an antigen the response to which is controlled by an Ir gene linked to the alloantigen against which the serum is directed. In contrast, when alloantisera are used with the parental strains a generalized non-specific inhibition of T cell proliferation is observed. Anti-B serum which is directed against the guinea pig equivalent of the murine D or K antigens also produces this non-specific generalized inhibition of T cell proliferation. This non-specific inhibition may result from a turn-off signal generated by the interaction of the alloantisera with the alloantigens on the T cell. Alternatively, part of the inhibition may be secondary to a generalize blockade of the interaction of antigen or mitogen pulsed macrophage with the sensitized T cell.

Project Subtitle IV: Role of the Macrophage in Genetic Control of the Immune Response

We have previously shown that the interaction of antigen pulsed macrophages with immune T lymphocytes requires that macrophage and T lymphocyte be syngeneic or semi-syngeneic. Although parental macrophages pulsed with an antigen not known to be under unigenic control were capable of activating F_1 T cells, parental macrophages obtained from an animal that lacked a given Ir gene were much less efficient in the activation of (responder x non-responder)F_1 T cell proliferation than either F_1 or responder macrophages. One explanation for these results is that the recognition sites for the antigen on the T cell are physically related to the sites for macrophage-T lymphocyte binding. According to this view, the non-responder macrophage functions poorly because the recognition sites for antigen are related to the macrophage-T lymphocyte binding site of the responder parent, while the main contacts between the F_1 lymphocyte and the macrophage of the non-responder parent are at the non-responder binding sites.

An alternative explanation for these experimental results is that macrophage-T lymphocyte interaction requires that the Ir gene product be expressed in the macrophage. In order to explore this question we have used populations of outbred animals where relationships between Ir genes and alloantigens exist which differ from those in the inbred populations. Thus, in inbred strain 13 animals and in the progeny of (2 x 13)F_1 x 2 backcross animals the genes controlling responsiveness to both GT and to low dose immunization with DNP-GPA are linked to the 13 alloantigen gene. Most outbred animals are 13^+ are also GT^+ and DNP-GPA^+; however, we have identified a small number of 13^+ GT+ DNP-GPA^- and 13^+ GT^- DNP-GPA^+ animals. We have used macrophages from these animals which bear the alloantigen in the absence of the linked Ir gene to activate T cell proliferation in cells derived from 13^+ GT^+ DNP-GPA^+ animals. The results of these studies demonstrated that macrophages from the 13^+ GT^- DNP-GPA^+ animals were incapable of activating T cell proliferation in cells from 13^+ GT^+ DNP-GPA^+ inbred strain 13 animals when pulsed with GT, but functioned normally when pulsed with DNP-GPA; in similar fashion, macrophages from 13^+ GT^+ DNP-GPA^- animals failed to activate inbred strain 13 cells when pulsed with DNP-GPA, but functioned relative normally when pulsed with GT.

Although these experiments appear to demonstrate that the alloantigen alone on the macrophage is insufficient to initiate T cell activation, further analysis of the alloantigens present on the cells of these animals demonstrated that cells from both the 13^+ DNP-GPA^+ GT^- and 13^+ DNP-GPA^- GT^+ animals were deficient not only in Ir gene product function but also lacked a specific alloantigen associated with that Ir gene product. Recently we have demonstrated that the 13 antigen associated with the GT Ir gene is distinct from the 13 antigen associated with the DNP-GPA Ir gene.

We next evaluated the situation where macrophage and T lymphocyte differ in their alloantigens, but appear to share the same Ir gene product. in these studies we used macrophages from animals that were 2^- but responded to the copolymer of L-glutamic acid, L-alanine (GA); inbred strain 2 animals are 2^+ GA^+. Combinations of 2^- GA^+ and 2^+ GA^+ macrophages and lymphocytes demonstrated significant T cell activation only when macrophage and T lymphocyte shared the appropriate alloantigen.

At the present time these studies are still incomplete. We have been unable to test whether the 2/13 antigens alone are responsible for macrophage-T lymphocyte interaction because we have failed to identify an animal that bears the full complement of alloantigen genes in the absence of the Ir gene that is normally associated with them. Studies in the GA system suggest that the Ir gene product alone is not the structure mediating macrophage-T lymphocyte interaction. However, it is still possible that both the Ir gene product and the alloantigen must be expressed in both the macrophage and T lymphocyte for effective cellular interaction. If this explanation proves correct it would be unlikely that the Ir genes would code for the specific antigen receptor of the T lymphocyte. In order to investigate this further we are studying the biochemical properties of the 2/13 antigens on the macrophage and studies are also being designed to investigate the possible association between antigen and alloantigen on the macrophage.

Most of the studies involving macrophage T lymphocyte interaction have been performed with the in vitro T lymphocyte proliferation assay. Because other types of T lymphocytes may have different histocompatibility requirements for activation, we have succeeded in developing an in vitro assay for antibody producing cells in the guinea pig. Thus far we have developed a reliable assay for plaque-forming cells using immune cells and have demonstrated that plaque-forming cells can be induced by antigen pulsed macrophages. The histocompatibility requirements for this interaction are still under investigation.

We have chosen the interaction of macrophage and T lymphocytes as a model for the interaction of two populations of immunocompetent cells. Further analysis of the requirements for this interaction on both a biological and chemical basis should provide insights into our understanding of the role of the products of the Ir genes in cellular interactions.

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1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Interactions of Lymphocytes in the Immune Response

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Cooperating Units: Dr. Richard Asofsky, LMI/NIAID; Dr. Michael Potter, LCBGY/NCI; Dr. John Wunderlich, IB/NCI; Dr. H.N. Eisen, Mass. Institute of Technology; Dr. N. Sakato, Mass. Inst. of Tech.; Dr. J. Kappler, Univ. of Rochester; Dr. P. Marrack, Univ. of Rochester; Dr. N. Klinman, Univ. of Pennsylvania; Dr. M. Julius, Stanford Univ.

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
TOTAL	2 1/4	1/4	1/4	2 3/4
Professional	1 1/4	1/4	1/4	1 3/4
Other	1	-	-	1

Project Description:

Keyword Descriptors: Thymus-derived lymphocytes; cellular interactions; immune response genes; cell receptors.

The object of the current investigations has been to understand how one class of immunologically competent cells, the thymus derived lymphocyte (or T cell), recognizes antigens and how it then proceeds to regulate responses of other cell types. In particular, emphasis has been placed on the role of T cells in regulating antibody responses, a function of thymus independent bone marrow derived lymphocytes (or B cells). T cells that augment antibody responses are called "helper" cells, while those that suppress such responses are called "suppressor" cells.

The first problem considered has been the specificity of antigen recognition by T cells. A system has been developed over the past two years in which guinea pig T cell responses to chemically defined antigenic determinants can be measured. This has allowed an inferential mapping of the antigen binding receptor in the T lymphocyte. These studies suggest that the T cell receptor displays an extraordinary precision in antigen recognition, and also that antigenic determinants recognized by individual T cells are larger and/or more complex than those recognized by B cells. Such receptors have been shown to be clonally distributed on T cells.

Another study on the precision of T cell antigen recognition carried out in collaboration with Drs. N. Sakato and H.N. Eisen of MIT, has shown that murine T cells discriminate individually specific antigenic determinants (idiotypes) of isologous myeloma proteins as accurately as do B cells or antibody. Such cells are interesting candidates for specific suppressor cells, and this possibility is being investigated in collaboration with Dr. N. Klinman at the University of Pennsylvania.

A number of studies on various aspects of helper cell function have been carried out. It has been shown that there may be two interacting specific helper cells, both probably T cells, in some antibody responses. These cells have not thus far been separated. However, further experiments appear to make their existence more likely. In particular, the only conflict in the experimental data has recently been shown to be due to the presence of a suppressive T cell, masking the activity of one of the putative two helper cells.

Another "helper" system involves helper effects mediated by serum antibody. Such effects have now been shown to require normal mature T cells. However, there is no evidence in such studies to support the contention that the antigen receptor on T cells is passively acquired serum antibody; indeed, these experiments argue strongly against such an hypothesis.

The role of genes of the major histocompatibility complex of the mouse in cellular interactions in antibody responses has also been studied, in collaboration with Dr. T. Werblin, Dr. W. E. Paul and R. Lieberman. Particular attention has been paid to the effect of those genes in the I region of the MHC which control antibody responses (Ir genes) on cellular interactions. It has been found that cells from mice sharing only the I-A subregion will collaborate effectively. Furthermore, by using antigens under genetic control, a carrier-specific failure to make an anti-hapten antibody response has been shown to be due, at least in part, to a defect of the hapten-specific B cells. This defect maps in the I-B subregion. This finding strongly suggests two types of Ir genes, one expressed on B cells and one on T cells. Both are specific for the "carrier". This concept has received support from the recent studies of Taussig, Benacerraf and Maurer and their colleagues.

Finally, a number of systems in which T cells act as suppressor cells have been discovered, usually inadvertently. A suppressor T cell may account for the resistance of unirradiated mice to syngeneic adoptive antibody responses. Adult thymectomy augments a variety of immune responses, which thymocytes suppress. Spleen cells cultured in vitro with or without alloantigens develop T cells that suppress both T-dependent and T-independent antibody responses, and T cell responses such as mixed lymphocyte reactions.

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ROCKY MOUNTAIN LABORATORY
HAMILTON, MONTANA
July 1, 1974 through June 30, 1975

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Summary Statement

Rocky Mountain Laboratory

Hamilton, Montana

July 1, 1974 through June 30, 1975

Administrative Report

Because of the number of senior staff retirements in the immediate future and the progressive decline in personnel ceiling, program planning for the next decade is of immediate importance to assure continuation of a viable dynamic research organization. Of 24 permanent doctorate level staff, 3 will face retirement for age and another 4 will be eligible to retire within 5 years. This problem was discussed at a number of staff meetings to explore alternatives of program direction that would accommodate and fully utilize existing staff and allow for the development of a reorganized program. Most thought our research program was too diverse and there was a consensus that future research should be oriented toward 2 major areas—1) arthropod- and animal-borne diseases, and 2) immunology and pathogenesis of chronic diseases. The idea was accepted that some reapportionment of facilities and technical support would be necessary to accommodate new professional staff that would be required for maintaining program strength.

During the past year, 11 appointments were made to the staff, including Dr. Michael Kelly and Dr. William Krinsky, Research Associates, and Dr. H. F. Hasenclever, who transferred from the Laboratory of Microbiology, NIAID. Fifteen employees were separated. Included in these were 2 employees who died from coronary thrombosis, 3 resignations, and 10 retirements. After Dr. James Brennan retired, the Medical Entomology and Acarology Section and the Zoonoses Section were combined into one section—Medical Zoology and Zoonotic Diseases Section, with Dr. J. F. Bell designated as Section Head. Dr. Donald Granger will complete his 2-year appointment as a Research Associate June 30, 1975.

PHS-NIH

Summary Statement

Rocky Mountain Laboratory

Hamilton, Montana

July 1, 1974 through June 30, 1975

Research Highlights

The following are highlights of research findings, of which many appeared in 44 published papers and 38 in press.

Rickettsioses

Marked interest in Rocky Mountain spotted fever (RMsf) has been stimulated by Dr. Burgdorfer's collaborative investigations with health departments and other agencies of states in the Southeast. Of 774 cases reported last year, 113 cases were recognized in North Carolina alone. From South Carolina, 56 physicians and others submitted 1,186 ticks for examination. Of these, 199 Dermacentor variabilis, 16 Amblyomma americanum, and 4 Rhipicephalus sanguineus had fed on man. The infection rate among D. variabilis, as in Alabama, was about 5%. To date, virulent Rickettsia rickettsii has not been recovered from A. americanum, a purported vector in the Southeast. (Burgdorfer, Krinsky)

Last year we thought that some eastern strains of R. rickettsii may be atypical in antigenic composition, because only one-third of patients, typically affected, developed complement-fixing antibodies. In further study of this problem, we examined paired sera from 97 patients probably affected with RMsf by complement-fixation (CF), microagglutination (MA), passive hemagglutination (HA), and micro-immunofluorescence (micro-IF) tests. The micro-IF test detected antibodies in 3 times as many patients as did the CF test, and the use of 10 units of antigen did not increase the sensitivity of the CF test. The micro-IF test is simple to perform and the antigen has remained stable for 3 years. A single drop of serum can be tested simultaneously against as many as 12 antigens. Antibodies detectable by this test appear about the 5th day of illness and reach peak titers by the 3rd week. Because of the antigen's stability and the simplicity of the test, it could easily be used in state health laboratories. Although the results of MA and HA tests are incomplete, both appear to be comparable in sensitivity to the micro-IF test. (Philip, Casper, Anacker, Burgdorfer, Thomas)

Results of further studies (serologic tests, guanine-cystosine analysis, vaccine cross protection, and mouse toxin neutralization) confirm earlier impressions that the organism repeatedly recovered from R. sanguineus is a new member of the spotted fever group. After challenge, dogs did not develop a rickettsia, but did develop antibodies that reacted in the MA test but not in the CF test. Apparently, dogs are not the reservoir animal for this organism. Whether this organism causes human disease has not been determined, but *R. sanguineus* in the United States does not commonly feed on man. (Burgdorfer, Krinsky, Anacker, Philip)

Studies were continued on the phase conversion that occurs when *Coxiella burnetii* is cultivated in embryonated eggs. Two of 3 cloned phase I strains (from a single organism) converted to phase II by the 20th yolk sac passage. Large inocula facilitated conversion, because genetic mutations that did occur were theoretically conserved. (Ormsbee)

Arthropod-borne viruses

To date, our investigations on the ecology of tick-borne viruses have shown that *Ixodes uriae* from Oregon to Alaska is commonly infected with viruses belonging to the Kemerovo, Sakhalin, and Uukuniemi groups. This tick species commonly feeds on seabirds and many, including newly hatched murres, are seropositive against one or more of the viruses, but all attempts to isolate these viruses from bird tissues have failed. This year we found that many unhatched embryonated eggs had antibodies, derived from the maternal yolk sac, to Tyuleniy and Yaquina Head virus. Hence, these birds would not likely circulate virus until maternally derived antibodies had cleared their bodies.

From soft-shelled ticks, we have isolated 3 strains of a new virus from *Argas brumpti* collected in Tanzania. Another new virus from *A. cooleyi* in North America has been characterized and registered as Sunday Canyon virus. From *Ornithodoros capensis* collected in Hawaii, we isolated Hughes virus for the first time in that area, and others, including Johnston Atoll and Midway.

Because of the general low pathogenicity of many tick-borne viruses for laboratory animals, we have continued to explore new systems for assaying antibody. We found that normal guinea pig serum has an accessory factor that increased neutralizing capacity of homologous antiserum by 10- to 100-fold, but not cross reactivity to heterologous strains. (Clifford, Yunker, Keirans, Thomas)

In vitro systems of vector and vector-related cells are being developed and applied to the study of arboviruses and rickettsias to better understand their relationships to vector cells. Differential virus-supporting and plaque-producing abilities of 7 lines of mosquito cells were found inferior, in most respects to Singh's *Aedes albopictus* cells. Production of syncytia and plaques in these cells by dengue viruses 1 to 4 was studied. Characteristic patterns were seen for each virus, titers of dengue-2 were consistently higher in mosquito than in Vero cells, and viruses were not separable by cross-plaque neutralization tests in mosquito cells. The suitability of mosquito cells for growth of certain arboviruses, such as Chikungunya, was improved by clonal selection from 10 populations of Ae. albopictus. The latent existence of 5 viruses in certain substrains of Ae. albopictus cells maintained by different laboratories was documented. The value of mosquito and Vero cell cultures for primary isolation and identification of certain rickettsias and wolbachias that are not readily studied in conventional egg or animal systems was demonstrated. A new mosquito cell-line (Ae. aegypti) was established in culture. PL-480 agreement 03-016-N (Tick biochemistry and physiology) was highly productive in the study of tick lipids, fatty acids, sterol metabolism, bloodmeal breakdown, haem synthesis, protein metabolism, juvenile hormone, water balance and respiration. Results are in press or published as 15 research papers. (Yunker)

Pertussis

In further efforts to characterize the histamine sensitizing factor (HSF) of Bordetella pertussis, we have determined that the purified component has at least 15 amino acids. Precipitin bands could not be shown by gel diffusion against hyperimmune rabbit serums, but neutralization of some of its activity by these serums indicates that it is antigenic. Pronase concentrations that fully digested control proteins did not affect HSF. Neither did ficin, trypsin, lipase, or papain affect its activity. Observations on the effect of epinephrine on the vascular permeability of muscle vessels induced by HSF indicate that β-adrenergic receptors may not be the major site of action of HSF. This conclusion is also supported by higher levels of cyclic AMP seen in lymphocytes of HSF-treated mice than in normal controls, even though the level can be increased in both by incubating cell suspensions in isoproterenol. (Munoz, Bergman)

Immunology of viral and neoplastic diseases

The major objectives of this group are to study immunological mechanisms of host defense against acute, persistent and latent viral and neoplastic diseases. Both systemic and local defenses are being investigated, with reference to both effector systems and characterization of target cell membrane antigenic composition.

Friend mouse leukemia virus. A single gene (RFV-1) which controls ability of mice to recover from Friend virus leukemia has been identified and mapped in the D region of the major histocompatibility locus (H-2). The immune response appears to be important in this recovery. Recovery correlates with the appearance of specific anti-leukemia cell cytotoxic T lymphocytes in the spleen. Virus proliferation appears to be controlled by neutralizing antibody in both recovering and persistently leukemic mice. (Chesebro, Bloom)

Aleutian disease virus of mink (ADV). ADV has been purified on CsCl gradients (density = 1.37) and visualized in the electron microscope (diameter = 25 nm). On the basis of density and morphology it is believed to be a parvovirus. Kinetics of anti-ADV antibody production has been followed in sapphire (susceptible) and pastel (resistant) mink. Both mink develop high antibody titers initially. Differences in susceptibility to the clinical immune complex disease are apparently not due to differences in anti-viral antibody production but may be a result of differences in viral proliferation in the 2 mink types. (Bloom, Chesebro)

Effects of specific and nonspecific immunity in virus infections. Continued studies with in vitro herpes simplex virus (HSV) infections have shown that activation by PPD of sensitized leukocytes obtained from CFA-inoculated rabbits significantly reduced HSV plaque numbers and plaque size. The interferon obtained by activation of sensitized leukocytes was shown to be highly efficient in that one leukocyte per 200 target cells inhibited HSV replication by >90%.

Recent experiments with vaccinia virus infections of rabbit skin have shown that an anti-viral mediator can be stimulated in rabbit skin by a specific immunological reaction, and that this mediator can be recovered in high titers. Stimulation of the mediator at the site of a previously established vaccinia skin infection results in a marked inhibition of virus replication early in infection, and almost complete inhibition of replication at that time when untreated lesions contain >6 logs of virus. (Lodmell, Bloom, Chesebro, Granger)

Role of Ig classes in immune defense mechanisms. Further studies on the immune response in the Syrian hamster have defined 2 cytophilic Ig-$7S\gamma_1$ and $7S\gamma_2$. A unique difference in their mode of action on macrophages is that $7S\gamma_1$ does not promote phagocytosis in contrast to a typical cytophilic Ig like $7S\gamma_2$. Furthermore, only $7S\gamma_1$ antibody was elicited after certain immunizations, which actually had a suppressing effect on $7S\gamma_2$ antibody production. For our studies on the curative or enhancing effect of antibody on tumor survival, we are using an SV-40 induced lymphoid tumor which has been found to synthesize a monoclonal Ig detected by idiotype specific antisera in serum and on the tumor cell membrane. (Coe, Portis)

Pathogenesis and immunology of rabies. Previously, we showed that elevated ambient temperature during the incubation period resulted in decreased mortality, frequent abortive infections, and delayed onset in mice infected with typical rabies virus. Essentially similar results were obtained with some temperature sensitive mutants; survival after onset was as high as 94% among mice held at high ambient temperature, versus 35% at normal temperature.

In a collaborative study with Dr. D. Blenden, University of Missouri, we have regularly demonstrated antibody-coupling rabies antigen in the skin of muzzle or facial sensory papillae in 45 experimentally infected and 5 naturally infected dogs. We are currently studying the early stages to determine when this antigen appears. The ability to demonstrate rabies antigen in the skin early in the disease will be extremely helpful in differentiating vaccinal paralysis from rabies. (Bell)

Immunology of tuberculosis

We have clearly shown interrelationships between pulmonary granuloma, delayed type hypersensitivity, and cell-mediated immunity in protection against tuberculosis induced by cell wall (CW) vaccines. Originally, it was thought that this immunity was specifically oriented by residual tubercular CW protein (PPD) when it was complexed with P3, a nonantigenic trehalose mycolate isolated from *Mycobacterium tuberculosis*. However, we now report that a nonrelated protein, such as acetylated bovine serum albumin (BSA), when combined with P3, induced protection against tuberculosis, which was consistent with the extent of pulmonary granuloma. Neither P3, PPD, nor acetylated BSA did so alone.

To evaluate the role of lymphocytes and macrophages in granuloma formation and correlated protection, we used silica, Con A, and corrageenan to influence their activity. These agents abrogated delayed hypersensitivity, but did not prevent pulmonary granuloma formation. When vaccinated mice were desensitized by multiple intravenous inoculations of BCG protoplasm, footpad delayed hypersensitivity, macrophage migration, and pulmonary granuloma formation, as well as protection, were suppressed. (Ribi, Granger, Kelly, Yamamoto)

Microbial constituents in cancer immunotherapy

Significant progress was made in our research on the role of various microbial fractions in cancer immunotherapy. CW and CW-skeleton preparations from *Mycobacterium phlei*, a fast growing saprophyte of group IV atypical mycobacteria were as effective in tumor regression as similar preparations from BCG, which requires 2 weeks cultivation for comparable yields. When preparations for either bacteria were combined with P3 in oil droplet vaccines, up to 90% of line 10 tumors in strain 2 guinea pigs were regressed. Similar cure rates were obtained when tumors were treated with oil droplet preparations containing only P3 and endotoxins, particularly the weakly pyrogenic extracts from rough (Re) mutants. These findings suggest that regression of tumors depends on the selective stimulation of cell-mediated immunity (without humoral response) directed first against the protein component of endotoxin or mycobacteria, and later to the tumor itself. The critical factor in specifically directing this immune response is the role of P3 in presenting the antigen. In some manner P3 irreversibly binds the antigen to the oil droplet. We have shown that this binding is dependent, in a linear fashion, on the relative concentration of P3, antigen, and oil. When fluorescein-conjugated BSA was used in the mixture, one could visualize fluorescent particles in or on the oil droplets. Without P3, they were dispersed in the aqueous phase. When too much oil was added, the mixture behaved like incomplete Freund's adjuvant and chiefly stimulated a humoral response. (Ribi, Granger, Milner, Kelly, Smith)

Pathogenesis of slow viral diseases

The development of encephalopathy in 7 of 8 groups of mink inoculated with brain suspensions from different naturally infected Suffolk sheep supports the theory that the natural disease in mink is caused by the ingestion of offal from scrapie-infected sheep. Mink similarly inoculated with brain suspension from one Targhee and 2 goats likewise became affected. Furthermore, mice inoculated with first passage scrapie virus in mink brain, after 20 months, developed clinically typical, but neuropathologically atypical, murine scrapie. Conversely, by 7 years after inoculation with mink encephalopathy virus, 6 of 19 sheep and 10 of 19 goats have slowly developed typical scrapie. It appears that a single passage in mink does alter scrapie virus and affects the type of disease subsequently caused in its natural host. These observations seem important in any comparisons of the related agents of Kuru, scrapie, mink encephalopathy and Creutzfeldt-Jakob disease.

Our recent studies have confirmed the existence of at least 4 strains of Aleutian disease virus that differ dramatically in their pathogenicity for mink of non-Aleutian genotype (pastels); all are highly pathogenic for the sapphire color phase (homozygous for Aleutian gene). When the weakest strain (Pullman) was titrated in pastels and sapphires, comparable endpoints were obtained, as measured by antibodies detectable by counter immunoelectrophoresis with Utah-1 antigen. However, all sapphires, but only a few pastels, succumbed. When surviving seropositive pastels were subsequently challenged with the most virulent strain (Utah-1), the disease was markedly more fulminating than that seen in previously unexposed pastels. These observations suggest that preexisting antibody is not protective, and the ability of pastels to overcome the initial infection is possibly due to factors that affect virus growth. (Hadlow, Bloom, Race, Chesebro)

PHS-NIH

Summary Statement

Rocky Mountain Laboratory

Hamilton, Montana

July 1, 1974 through June 30, 1975

Honors and Awards

The following honors and outside service reflect the scientific recognition afforded staff of the Rocky Mountain Laboratory.

Awards:

Dr. W. Burgdorfer - DHEW Superior Service Award, May 1974
Dr. H. G. Stoenner - K. F. Meyer Gold Headed Cane Award, July 1974

Editorial Boards of Journals:

Dr. J. Munoz - Infection and Immunity
Dr. E. Ribi - Infection and Immunity
Dr. H. G. Stoenner - American Journal of Veterinary Research
Dr. C. E. Yunker - Journal of Medical Entomology

Five additional staff members and 2 of the above regularly review manuscripts for Journal of Parasitology, Journal of Immunology, Annals of Entomological Society of America, Journal of the National Cancer Institute, American Review of Respiratory Diseases, and Journal of Clinical Investigations.

Society Posts:

Dr. L. A. Thomas - President, International Northwest Conferences on Diseases of Nature Transmissible to Man
Dr. E. Ribi - Chairman, U.S.-Japan Tuberculosis Panel
Dr. H. Hasenclever - Chairman, Standards and Examining Committee, Medical Mycology. Member, American Board of Medical Microbiology
Dr. H. G. Stoenner - Chairman, Leptospirosis Committee, U.S. Animal Health Association

Invited Lectures and Participation in Symposia:

The following staff—Drs. E. Ribi, C. E. Yunker, J. J. Munoz, and W. Burgdorfer—participated in 6 international congresses in fields of their special research interests.

In addition, the following staff—Drs. H. Hasenclever, W. Burgdorfer, D. Granger, M. T. Kelly, R. N. Philip, R. Anacker, E. Ribi, B. Chesebro, C. E. Yunker, and H. G. Stoenner—presented 22 lectures at universities or participated in national symposia.

Project No. Z01 AI 00061-13 RML
1. Rocky Mountain Laboratory
2. Rickettsial Diseases
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Natural history of tick-borne rickettsial agents and their public health significance

Previous Serial Number: NIAID-171

Principal Investigator: W. Burgdorfer

Other Investigators: R. L. Anacker, L. P. Brinton, R. K. Gerloff, R. N. Philip, and L. A. Thomas

Cooperating Units:
Dr. T. Adkins, Jr., Clemson University, Clemson, N.C.
Dr. J. L. Benach, State of New York Dept. of Health, White Plains
Dr. J. R. Burk, Alabama Dept. of Public Health
Mr. Hicks, Jefferson County Health Dept., Birmingham, Alabama
Captain Keyt, Madigan Army Medical Center, Tacoma, Washington
Dr. B. Norment, Mississippi State University, State College, Miss.
North Carolina Dept. of Human Resources, Division of Health Services, Raleigh: Dr. N. J. MacCormack and Dr. D. J. Sexton
South Carolina Dept. of Health and Environmental Control, Columbia: Mr. S. M. Loving and Dr. L. E. Priester

Man Years:

Total:	5 10/12
Professional:	2 4/12
Other:	3 6/12

Project Description:

This project pertains to the spotted fever problem in the United States and studies related to the ecology of the etiologic agent, Rickettsia rickettsii, and of closely related tick-borne rickettsiae.

Emphasis is placed in providing directions and support to outside agencies (state health departments, public health laboratories, hospitals, universities, etc.) in their own educational programs and surveys, and in training their personnel in recently developed diagnostic procedures.

1. The successful South Carolina campaign initiated in 1973 to inform the public and medical practitioners about spotted fever and the ticks that transmit *R. rickettsii* was terminated. As a result of the response by the public and medical profession (56 physicians participated), 1,186 ticks consisting of 987 *Dermacentor variabilis*, 103 *Rhipicephalus sanguineus*, and 97 *Amblyomma americanum* were examined. Rickettsiae identified by direct immunofluorescence as members of the spotted fever group were detected in 49 (4.9%) *D. variabilis*, and 16 (16.6%) *A. americanum*. Two hundred and twenty (199 *D. variabilis*, 17 *A. americanum*, and 4 *R. sanguineus*) had been attached to 199 persons. Nine of these ticks (8 *D. variabilis* and one *A. americanum*) were hemolymph test-positive for spotted fever group rickettsiae.

As a result of this study and because the public in South Carolina responded so enthusiastically to the program, the Department of Health and Environmental Control in Columbia, South Carolina, is now conducting its own educational and tick examination program. To familiarize the personnel with the technical aspects of the program, particularly the hemolymph test, a workshop was held in Columbia.

2. In cooperation with Dr. J. Burk of the State of Alabama Department of Public Health, a survey was conducted throughout 10 Alabama counties to determine the prevalence of ticks in that area. Tick collections were made only from dogs submitted to animal clinics. Twenty-three (3.4%) of 794 *D. variabilis*, 4 (18.2%) of 27 *A. americanum*, one (1.7%) of 59 *A. maculatum*, and one (0.2%) of 574 *R. sanguineus* were infected. The largest percentages of infected *D. variabilis*, namely 7.8%, 4.5%, and 3.3%, were recorded in Morgan, Etowah, and Jefferson counties where numerous spotted fever cases in man occur annually.

3. As a result of the wide publicity afforded the South Carolina campaign and the usefulness of the hemolymph test, health institutions of several eastern and southeastern states have already initiated—or are planning to initiate—educational programs and surveys in areas of high disease incidence. In the following instances, RML's assistance and collaboration has been solicited:

a. Plans have been made for a collaborative study with the Division of Health Services of the Department of Human Resources in North Carolina, where 113 Rocky Mountain spotted fever (RMsf) cases were recorded in 1974, to determine the prevalence of spotted fever rickettsiae-infected ticks in certain areas of that state.

b. A similar study will be initiated on Long Island where, in 1974, 31 cases of RMsf occurred.

c. A tick/rickettsial survey will be conducted also at the U.S. Army's Yakima Firing Center in central Washington where many troops are exposed to large tick populations.

4. Results of further studies (serologic tests, biochemical analyses, vaccine potency and mouse neutralization tests) with the rickettsia isolated from *R. sanguineus* off dogs in Mississippi, indicate that this agent is a new member of the spotted fever group. Additional isolates indistinguishable from this agent have been made from *R. sanguineus* collected in Alabama and North Carolina, and from a *D. variabilis* off a patient in South Carolina.

Although dogs develop MA antibodies (but not CF) against this agent, they did not develop a rickettsemia from feeding by infected *R. sanguineus*, or injection of infected yolk-sac, tissue culture, or tick material. This suggests that dogs are not the reservoir animals for infecting ticks. Studies are in progress at the Mississippi State University to clarify the biology of *R. sanguineus*, particularly to establish the host range of the immature stages of this tick in that area. In addition, a project has been initiated to evaluate the behavior of eastern strains of *R. rickettsii* in dogs and in *R. sanguineus*.

5. Research pertaining to the rickettsia-like, nonpathogenic microorganisms (see last Annual Report) isolated from *D. andersoni*, *D. variabilis*, and *A. americanum* was continued. Several isolates have been established in mammalian cell cultures (see No. Z01 AI 00066-02 RML), and purified antigens have been prepared for studies of their relationship to pathogenic rickettsiae (see No. Z01 AI 00065-02 RML).

Keyword Descriptors: Rocky Mountain spotted fever, eastern and southeastern U.S., tick surveys, hemolymph test, *Rickettsia rickettsii*, rickettsia-like organisms, dogs

Publications:

Burgdorfer, W.: Introduction to rickettsioses, Q fever, rickettsialpox, Rocky Mountain spotted fever, murine (flea-borne) typhus fever, boutonneuse fever, Queensland tick typhus, North Asian tick typhus, scrub typhus. In Hubbert, W. T., McCulloch, W. F., and Schnurrenberger, P. R. (Eds): *Diseases Transmitted from Animals to Man* (ed. 6). Springfield, Ill., Charles C Thomas, 1975, pp. 382-429.

In press:

Burgdorfer, W.: Spirochetal diseases. The relapsing fevers. In Hunter, G., Frye, W. W., and Swartzwelder, J. C. (Eds.): Manual of Tropical Medicine (ed. 5).

Burgdorfer, W.: Rickettsial diseases (introduction, epidemic (louse-borne) typhus, murine (flea-borne) typhus, rickettsialpox, Queensland tick typhus, trench fever). In Hunter, G. W., Frye, W. W., and Swartzwelder, J. C. (Eds.): Manual of Tropical Medicine (ed. 5).

Burgdorfer, W.: Current status of tick-borne typhus (Rocky Mountain spotted fever) in the United States. Proc. IV International Congress of Acarology, Saalfelden, Austria, Aug. 12-19, 1974.

Burgdorfer, W. and Brinton, L. P.: Mechanisms of transovarial infection of spotted fever rickettsiae in ticks. Ann. N.Y. Acad. Sci.

Burgdorfer, W., Adkins, T. R., Jr., and Priester, L. E.: Rocky Mountain spotted fever (tick-borne typhus) in South Carolina: an educational program and tick/rickettsial survey in 1973 and 1974. Am. J. Trop. Med. Hyg.

Burgdorfer, W.: A review of Rocky Mountain spotted fever (tick-borne typhus), its agent, and its tick vectors in the United States. J. Med. Entomol.

Burgdorfer, W., Sexton, D. J., Gerloff, R. K., Anacker, R. L., Philip, R. N., and Thomas, L. A.: *Rhipicephalus sanguineus*: vector of a new spotted fever rickettsia in the United States. Infect. Immun.

Sexton, D. J. and Burgdorfer, W.: Clinical and epidemiologic features of Rocky Mountain spotted fever in Mississippi, 1933-1973. South. Med. J.

Project No. Z01 AI 00062-06 RML
1. Rocky Mountain Laboratory
2. Rickettsial Diseases
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cellular and subcellular aspects of vector-pathogen relationships

Previous Serial Number: NIAID-186

Principal Investigator: L. P. Brinton

Other Investigators: W. Burgdorfer and W. L. Krinsky

Cooperating Units: Dr. C. W. Ng, Bureau of Virology, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada

Man Years:

Total:	2
Professional:	1 1/12
Other:	11/12

Project Description:

This project is primarily concerned with cellular and subcellular changes induced by etiologic agents (viral, rickettsial, bacterial, and protozoal) of human disease, including microorganisms of actual or potential zoonotic significance, in their arthropod and vertebrate vector-host reservoirs. Electron microscopic (EM) techniques are applied to characterize subcellular structures of normal and infected arthropod tissues and to determine the cytodifferentiation and cytomorphology of microorganisms during intracellular development.

Efforts to obtain ultrathin sections of phlebotomine sandflies infected with vesicular stomatitis virus (VSV) have consistently failed. Our supply of infected tissues, prepared for electron microscopy, has essentially been exhausted. Studying the development of VSV interaction with cells of different organ systems following bloodfeeding and during transovarial passage would provide a clearer understanding of vector-pathogen associations.

Studies were continued on the morphogenesis and localization of Colorado tick fever (CTF) virus in tissues of Dermacentor andersoni. CTF virus has been visualized in hypodermal and salivary gland cells of infected female ticks. Virions were scarce in salivary gland and were present in only slightly greater numbers in hypodermal cells. A better understanding of CTF virus replication and localization in its primary vector is important, as it relates to the efficiency with which the vector transmits virus.

A comparative fine structure study of Wolbachia-like symbiotes in D. andersoni, Rhipicephalus sanguineus, and Haemaphysalis leporispalustris is nearing completion. Symbiotes in R. sanguineus are the largest forms observed in these 3 species of ticks and possess a distinct cell wall, which is indistinct or lacking in symbiotes of the other 2 tick species. The presence of Wolbachia-like symbiotes in tick vectors of viral and rickettsial agents of human disease provides a valuable opportunity to compare growth characteristics of nonpathogenic vs pathogenic microorganisms at the cellular and subcellular level. The symbiote in H. leporispalustris resembles the suckling mouse cataract agent reported to be isolated by Ziegel and Clark (1975) from this tick species.

Preparations are being completed for a comparative fine structure study in Vero cells of Rickettsia rickettsii and the recently isolated Rickettsia sp. from R. sanguineus ticks from Mississippi. Because the R. sanguineus agent may cause human disease, a comparative study of these rickettsial species is necessary to establish similarities and differences between these organisms.

Fine structure studies on the cytomorphology of the 277-F agent isolated from H. leporispalustris in 1967 reveals that this organism is closely similar to a mycoplasma-like agent belonging to the recently described genus Spiroplasma. Our 277-F agent is remarkably similar in fine structure to S. citri as described by Cole et al. (1973) in the Journal of Bacteriology. Fine structure of the 277-F agent cultivated in chick embryo allantoic fluid and in the central nerve mass of Ornithodoros moubata ticks will be described in the near future.

Keyword Descriptors: Cell biology (cytodifferentiation, cytomorphology, and cytochemistry) of vector-pathogen relationships

Publications:

Brinton, L. P., Burgdorfer, W., and Oliver, J. H., Jr.: Histology and fine structure of spermatozoa and egg passage in the female tract of Dermacentor andersoni Stiles (Acari-Ixodidae). Tissue Cell 6: 109-125, 1974.

In press:

Burgdorfer, W. and Brinton, L. P.: Mechanisms of transovarial infection of spotted fever rickettsiae in ticks. Ann. N.Y. Acad. Sci.

Krinsky, W. L.: Cystic anomaly in the intestine of adult Tabanidae. J. Invertebr. Pathol.

Project No. Z01 AI 00063-05 RML
1. Rocky Mountain Laboratory
2. Rickettsial Diseases
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Immune responses to rickettsial infections

Previous Serial Number: NIAID-171

Principal Investigator: R. A. Ormsbee

Other Investigators: R. N. Philip

Cooperating Units: None

Man Years:

Total:	2 7/12
Professional:	9/12
Other:	1 10/12

Project Description:

Antibody responses to natural infections and to experimentally administered antigens have been studied in the course of efforts 1) to improve diagnosis and prevention of rickettsial disease, 2) to improve understanding of antigenic relationships of pathogenic rickettsiae, and 3) to study the differences between Coxiella burnetii and all other pathogenic rickettsiae.

We reported last year on the occurrence of spotted fever group antibodies in patients with epidemic typhus fever. This year we report finding typhus group antibodies in patients convalescent from Rocky Mountain spotted fever. It appears that humans and monkeys have capacity to respond to rickettsial infections with cross-reacting antibodies not found in experimentally infected goats, sheep, guinea pigs, and hamsters.

Preliminary tests of spotted fever and epidemic typhus vaccines by a method previously developed for Q fever vaccine and based on fever reduction in vaccinated guinea pigs have given reproducible results. This method promises to become useful for protective potency evaluation of typhus and spotted fever vaccines.

In response to the WHO program on evaluation of diagnostic tests, we extracted with NaCl soluble antigens from 5 species of the spotted fever group. These antigens, stored at 4° C, have remained stable and usable as diagnostic reagents in the complement fixation test for over 4 months. They have not become anticomplementary.

Although a close antigenic relationship exists between *Rickettsia prowazekii* and *R. canada*, we have found that the latter can be differentiated from all other members of the typhus and spotted fever groups by its relative resistance to the antibiotic activity of streptomycin in tissue cultures of chick embryo fibroblasts.

Keyword Descriptors: antibody, antigen, rickettsial, diagnosis, pathogen, typhus, spotted fever, man, vaccine

Publications: None

Project No. Z01 AI 00064-15 RML
1. Rocky Mountain Laboratory
2. Rickettsial Diseases
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanisms of phase conversion in Coxiella burnetii, causative agent of Q fever

Previous Serial Number: NIAID-171

Principal Investigator: R. A. Ormsbee

Other Investigators: None

Cooperating Units: Dr. Paul Fiset, University of Maryland, School of Med., Baltimore

Man Years:

Total:	5/12
Professional:	3/12
Other:	2/12

Project Description:

Coxiella burnetii differs from other rickettsiae in that it undergoes a change, phase I→phase II, on cultivation in chick embryos and may undergo the reverse, phase II→phase I, in animals. The phase shift is characterized by loss or gain of phase I antigen, among other changes. Phase I antigen has unique immunogenic and adjuvant capabilities which make it of continuing interest immunologically.

Phase I→phase II conversion occurred gradually in 2 of 3 cloned strains by the 20th yolk sac passage. Conversion is facilitated by a large inoculum, which theoretically should help conserve any phase I→phase II genetic mutations which might occur. A large inoculum should exert little effect on phase conversion caused by a mechanism other than mutation. We believe a phase I→phase II mutation occasionally occurs and is conserved in our yolk sac culture system.

In a search for recrudescent human perinatal infections with *C. burnetii* we have so far been unable to recover the organism from placental tissue or fetal blood in cases in which an elevated antibody level against *C. burnetii* has been found. The effort is continuing. (Collaboration with Dr. P. Fiset).

Keyword Descriptors: *Coxiella*, rickettsiae, phase, antigen, mutation, recrudescent, infection, perinatal, conversion

Publications: None

Project No. Z01 AI 00065-02 RML
1. Rocky Mountain Laboratory
2. Rickettsial Diseases
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Antigens and classification of the rickettsiae

Previous Serial Number: NIAID-171

Principal Investigators: R. L. Anacker and R. N. Philip

Other Investigators: W. Burgdorfer, E. A. Casper, R. K. Gerloff, and L. A. Thomas

Cooperating Units: Dr. C. W. Ng, Bureau of Virology, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada

Man Years:

Total:	4 7/12
Professional:	2 2/12
Other:	2 5/12

Project Description:

A variety of approaches is being utilized to identify and characterize rickettsial antigens and to aid in classification of rickettsiae.

Methods were developed to purify Rickettsia rickettsii strain R by centrifugation in a linear sucrose gradient in a zonal rotor, and various properties of the purified rickettsiae were investigated. Doses of 0.005-0.02 μg reduced fever in vaccinated guinea pigs to half that observed in control animals after experimental infection, whereas 35 μg of commercial vaccine were required to achieve the same degree of protection. Doses of 0.002-0.003 μg protected 50% of the mice from the lethal effects of 2 LD_{50} of R. rickettsii toxin as did 13 μg of commercial vaccine. Lyophilization of the purified rickettsiae reduced vaccine potency by as much as 2 logs.

Purified R. rickettsii was used as antigen in microagglutination (MA), hemagglutination (HA), and complement fixation (CF) tests in titrations of 31 human sera sent to RML for serological tests. Antibody was detected in 38.7% with the standard CF test using conventional, ether-extracted yolk-sac antigen, 9.7% with the CF test employing purified antigen, 45.2% with the MA test, and 87.1% with the HA test. Purified antigen probably failed to detect CF antibody in most of the sera because group antigen was almost completely removed during the purification procedure.

The above results suggest that zonal centrifugation may be a valuable procedure for the preparation of *R. rickettsii* vaccine and diagnostic reagent. Purified rickettsiae protected guinea pigs and mice at least 2,000 times better than did commercial vaccine. Also, our preliminary results indicated that the HA test may permit earlier diagnosis of Rocky Mountain spotted fever.

A study was begun to determine DNA composition of rickettsial agents isolated from several species of ticks collected either in Montana or in the eastern part of the United States. Although there is a serological cross-reaction between *R. rickettsii* and an agent isolated from the tick *Rhipicephalus sanguineus*, differences in their guanine-cytosine content indicated that these organisms represented 2 different species. This study should provide an objective aid to the more conventional methods for comparison and classification of rickettsiae.

A micro-immunofluorescence (IF) test whereby the same serum dilutions can be tested against as many as 12 antigens simultaneously is being used to examine antigenic relationships among spotted fever- and typhus-group rickettsiae in cross-tests of mouse antisera.

Antigens and antisera were prepared from strains previously characterized in CF, mouse toxin-neutralization and immunization experiments as representative of 8 species of spotted fever-group rickettsiae (*R. rickettsii*, *R. conorii*, *R. akari*, *R. australis*, *R. sibirica*, *R. montana*, *R. parkeri* and the *Rhipicephalus sanguineus* agent) and 3 species of typhus-group rickettsiae (*R. prowazekii*, *R. typhi* and *R. canada*).

Specificity and patterns of heterologous reaction among spotted fever-group rickettsiae were quite similar to those obtained by toxin neutralization in mice.

Four major groups of strains were identified which did not cross-react by IF. Included in each group were: 1) *R. rickettsii*, *R. parkeri*, *R. sibirica*, *R. conorii*, *R. montana*, *R. australis* and the *Rhipicephalus sanguineus* agent; 2) *R. akari*; 3) *R. prowazekii* and *R. typhi*; 4) *R. canada*. It is noteworthy that this sharp distinction between groups is not usually evident in IF tests of human sera.

Differences of varying degree were also noted within groups. For example, some sharing of antigens was evident for R. rickettsii and R. parkeri, but both organisms were only remotely related to R. montana. On the other hand, R. australis was completely distinct from all members of the group except R. montana. R. typhi, although closely related, could be differentiated from R. prowazekii.

These preliminary observations suggest the possibility that this serologic system may be useful for classification of rickettsiae. We are currently preparing antigens and antisera from spotted fever-group strains isolated from a variety of sources in this country for testing by IF to determine if systematic grouping is feasible.

Keyword Descriptors: antigen, classification, DNA, hemagglutination, immunofluorescence, rickettsiae, Rickettsia rickettsii, serotyping

Publications:

Anacker, R. L., Gerloff, R. K., Thomas, L. A., Mann, R. E., Brown, W. R., and Bickel, W. D.: Purification of Rickettsia rickettsi by density-gradient zonal centrifugation. Can. J. Microbiol. 20: 1523-1527, 1974.

In press:

Anacker, R. L., Gerloff, R. K., Thomas, L. A., Mann, R. E., and Bickel, W. D.: Immunological properties of Rickettsia rickettsii purified by zonal centrifugation. Infect. Immun.

Project No. Z01 AI 00066-02 RML
1. Rocky Mountain Laboratory
2. Epidemiology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Growth characteristics of rickettsiae in cell cultures

Previous Serial Number: NIAID-171

Principal Investigator: R. K. Gerloff

Other Investigators: R. L. Anacker, W. Burgdorfer, and R. N. Philip

Cooperating Units: None

Man Years:

Total:	3
Professional:	1 7/12
Other:	1 5/12

Project Description:

The objectives are 1) to determine the growth capabilities of various rickettsiae in cell cultures, 2) to determine optimal conditions for propagating rickettsiae in cell cultures, 3) to find cultural characteristics which might aid differentiation and classification of rickettsiae, and 4) to produce and purify cell culture-grown rickettsiae in sufficient quantity for use in serologic and immunologic studies.

Standard tissue culture procedures are employed except that centrifugation is used extensively to enhance infection of cell monolayers. Purification utilizes combinations of differential centrifugation, ultrasonication, and enzymatic digestion. The following rickettsiae have been studied extensively:

Rickettsia rickettsii
1. R (from *Dermacentor andersoni*)
2. TVA (from *D. variabilis*)
3. 275F (from *D. andersoni*)

R. montana
4. M-5-6-B

R. conorii
5. Kenya tick typhus (from *Haemaphysalis leachii*)

Unclassified

6. *parumapertus* agent
7. *Rhipicephalus sanguineus* agent
8. WB-8-2 from *Amblyomma americanum* (a member of the spotted fever group)

Mouse L cells were a satisfactory host for Nos. 1, 3, 5, and 7. Vero cells were preferred for the other 4 strains. Three other strains of R. rickettsii, namely the Wachsmuth, Hanson and Singleton, are currently under study and it appears that all three grow in L cells. The preferred temperature for growth of strain 7 has been 36.5° C; for all others, 33.5°. Medium 199 with 2% bovine fetal calf serum and 5 units penicillin per ml has been a very satisfactory cell maintenance medium in all instances.

Of the various strains, the cytopathic effects of No. 1 are clearly the most severe and most complete. In a titration, infection by even the smallest dosage always results in eventual complete destruction of an L cell monolayer. The effects of strain 5 are similar, both qualitatively and quantitatively. Several of the other strains behave in a completely different manner, growing abundantly within host cells yet not visibly damaging them.

Several batches of packed L cells heavily infected with strain 1 or 7 have been prepared for collaborative studies with Dr. Anacker. Concentrated suspensions of strains 2, 3, 4, 5, 6, 7 and 8 in either L or Vero cells have been supplied to Dr. Philip. A few batches of purified agent 7 have been supplied to Dr. Burgdorfer. The separate reports of these 3 investigators should be consulted for findings.

Study of growth of strain 1 in suspended L cells was continued. In the most satisfactory combination of conditions, the concentration of live host cells was maintained at 2 X 10^6 per ml. The required number of cells for a given volume of culture was mixed with sufficient rickettsiae to provide a 2:1 multiplicity of infection. Cells and rickettsiae were concentrated 15-fold for an initial infection period of 1 hr. The mixture was then diluted to full volume in a conventional spinner flask. At 48-hr intervals thereafter, half of the culture was harvested, a sample was frozen for subsequent titration of infectivity, and another was used for determination of the number of live cells remaining. Fresh cells from a normal spinner culture were added to restore the number of live cells to 2 X 10^6 per ml. Usually this required the equivalent of about 1.25 X 10^6 per ml.

Infected cultures were routinely maintained 12 to 14 days, during which the suspension at harvest usually contained 10^7 to $10^{7.5}$ $TCID_{50}$ of rickettsiae per ml. (Titers might have been higher if samples could have been titrated without prior freezing.)

Observation of marked differences in growth of strain 1 in individual L cells following exposure of monolayers to large inocula suggested great variation in innate susceptibility of cells. Cloning of cells is now being attempted in an effort to establish sub-lines which will provide greater yields of rickettsiae than will the parent line.

Experience of recent years has indicated that the chick embryo yolk sac is not optimal for propagating all strains of rickettsiae which exist in nature; some grow better in cell cultures. Besides, cell cultures offer greater opportunity for study of intracellular growth and pathologic changes, "cleaner" material for antigen preparation, and more criteria for rickettsial differentiation and classification. This project attempts to contribute to knowledge of rickettsiae by utilizing these advantages of cell cultures.

Keyword Descriptors: rickettsiae, growth, cell, tissue, culture

Publications:

Reeve, P., Gerloff, R. K., Casper, E., Philip, R. N., Oriel, J. D., and Powis, P. A.: Serological studies on the role of Chlamydia in the aetiology of non-specific urethritis. Br. J. Vener. Dis. 50: 136-139, 1974.

Project No. Z01 AI 00067-11 RML
1. Rocky Mountain Laboratory
2. Medical Zoology and Zoonotic Diseases
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Tularemia

Previous Serial Number: NIAID-173

Principal Investigator: J. F. Bell

Other Investigators: None

Cooperating Units: Fukushima Medical College, Fukushima, Japan:
Drs. M. Kojima, N. Kusunoki, and Y. Yamagata
Ohara General Hospital, Fukushima, Japan:
Drs. S. Ohara, T. Saito, H. Shiba, and T. Ueno

Man Years:

Total:	1
Professional:	2/12
Other:	10/12

Project Description:

Water-borne tularemia is known to cause extensive epidemics over wide areas of the Northern Hemisphere and surveys based on immune reactions attest that the disease is much more prevalent than is generally recognized. Neither the source nor the mechanism whereby Francisella tularensis survives for long periods in lotic waters has been explained. We have found that chronic nonfatal tularemia with nephritis and bacteriuria can be produced in voles by ingestion of fully virulent organisms (a common cause of infection among them) although one bacterium of the same strain inoculated parenterally will uniformly cause fatal infection. Cricetid rodents are ubiquitously associated with contaminated waters and epizootic tularemia among them is undoubtedly responsible for surges in water contamination. Demonstration of persistent bacteriuria in voles affords a logical explanation of chronic contamination of waters. The pyelonephritis produced by ingestion is almost certainly hematogenous in origin and, therefore, contradictory to current consensus regarding the pathogenesis of that kind of nephritis. A description of the conditions causing nephritis and bacteriuria in voles has been accepted for publication. A report on histopathology of the nephritis is being prepared (in collaboration with pathologists in Japan). A wide and stable gradient of susceptibility to the ingested bacteria was revealed in these experiments. No explanation is evident.

Keyword Descriptors: tularemia, rodents, pyelonephritis, bacteriuria, waterborne

Publications:

Owen, C. R.: Genus *Francisella* Dorofe'ev 1947, 176. In Buchanan, R. E. et al. (Eds.): *Bergey's Manual of Determinative Bacteriology* (ed. 8). Baltimore, The Williams and Wilkins Co., 1974, pp. 283-285.

In press:

Bell, J. F. and Stewart, S. J.: Chronic shedding tularemia nephritis in rodents: possible relation to occurrence of *Francisella tularensis* in lotic waters. *J. Wildlife Dis*.

Project No. Z01 AI 00068-15 RML
1. Rocky Mountain Laboratory
2. Medical Zoology and Zoonotic Diseases
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Rabies

Previous Serial Number: NIAID-173

Principal Investigator: J. F. Bell

Other Investigators: None

Cooperating Units: Dr. Donald Blenden, University of Missouri, Columbia
Dr. H. F. Clark, Wistar Institute, Philadelphia, Pa.
Dr. D. P. Perl, Brown University Medical School, Providence, R.I.

Man Years:

Total:	1 8/12
Professional:	9/12
Other:	11/12

Project Description:

Effects of high ambient temperatures on various stages of rabies virus infection in mice were studied. Ambient temperature, increased within the tolerated range, was found to have little effect upon body temperature of normal mice but caused marked elevation of temperature during illness. Increased body temperature was associated with decreased mortality, frequent abortive infections, and delayed onset, but exposure to high ambient temperature after onset of patent illness did not affect the course of the disease (published 1974). Similar experiments were done with several temperature sensitive mutants and with some rabies-related viruses with Dr. H. F. Clark, Wistar Institute. Differences in gross mortality between mice in high and low temperature ambiences were as great as 5% vs 58%, respectively. Survival after onset of illness was as high as 94% at high temperature compared with 35% at "normal" temperature. Some of the mutants reacted similarly; others showed much less striking differences.

Dr. Donald Blenden has demonstrated fluorescent antibody (rabies)-coupling antigen in skin, especially hair follicles, of a large percentage of rabies-infected animals. All of 45 cases of experimental rabies and 5 terminal natural infections in dogs have had demonstrable antigen in skin of muzzle, or facial sensory papillae. Skin of one dog had antigen on the day of onset. Muzzle skin of 3 cattle and one cat and skin of 4 humans with confirmed rabies contained antigen. Studies have been handicapped by lack of rabies-infected dogs and cats in early stages of infection, and we have furnished the desired animals. Studies of persistence of antigen in the skin have been possible because we have furnished animals in all stages of recovery.

Electron microscope and histopathologic studies of recrudescent rabies in a cat have been completed with Dr. D. P. Perl, Brown University, and will be submitted for publication. Several other cats and dogs that have recovered from rabies are in various stages of study. Attempts to activate possible latent infection in them have failed. It is noteworthy that an originally wide disparity between antibody titer in serum and cerebrospinal fluid has become much narrower in the several years of observation. Persistence of antibody and especially increased antibody titers are usually regarded as evidence of persistent antigenic stimulus. We believe that most available evidence supports a concept that antibody production in the central nervous system is autonomous. We are planning a workshop on central nervous system immunity to be cosponsored by RML and Rutgers University (Walter Schlesinger).

In September 1974, Dr. Glen C. Halver, Veterinarian, Administrator of the Animal Health Division of the Montana Department of Livestock, and J. F. Bell were co-chairmen of a Regional International Sylvatic Rabies Conference in Billings, Montana. From that conference a working organization was formed to coordinate activities on rabies investigation and control between Canadian and U.S. federal, provincial and state agencies (public health, livestock, and fish and game).

Keyword Descriptors: hyperthermia, ambience, latency, survival, recrudescence, cerebrospinal fluid, antibodies, sylvatic, skin, antigen

Publications:

Bell, J. F. and Moore, G. J.: Effects of high ambient temperature on various stages of rabies virus infection in mice. Infect. Immun. 10: 510-515, 1974.

Bell, J. F. and Munoz, J. J.: Enhancement of immune response to rabies virus by *Bordetella pertussis* extract. In Regamey, R. H., Hennessen, W., Lang, R., Perkins, F. T., and Triau, R. (Eds.): *International Symposium on Rabies, Lyon 1972; Symp. Series immunobiol. Standard*. Basel, S. Karger AG, 1974, vol. 21, pp. 199-206.

In press:

Bell, J. F.: Latent and abortive rabies. In Baer, G. M. (Ed.). *The Natural History of Rabies*. New York Academic Press.

Project No. Z01 AI 00069-44 RML
1. Rocky Mountain Laboratory
2. Medical Zoology and Zoonotic Diseases
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Systematics and vector relationships of certain parasitic arthropods

Previous Serial Number: NIAID-174

Principal Investigator: J. E. Keirans

Other Investigators: C. M. Clifford, C. E. Yunker, and J. M. Brennan

Cooperating Units: Miss Mona Al Taqi, University of Kuwait, Kuwait
Dr. Omar M. Amin, Univ. of Wisconsin-Parkside, Kenosha
Brigham Young University, Provo, Utah: Mr. J. T. Reed, Mr. R. Saunders, Dr. Selby Herrin
Dr. A. Canaris, University of Texas, El Paso
Mrs. M. B. Chitwood, Primate Research Center, Univ. of California, Davis
Dr. Emmett R. Easton, Central Veterinary Lab., Dar es Salaam, Tanzania
Dr. J. A. Hair, Oklahoma State University, Stillwater
Dr. T. M. Hefnawy, Cairo University, Cairo, A.R.E.
Dr. R. E. Hopkins, Travenol Labs., Morton Grove, Ill.
Dr. K. M. Howell, Univ. of Dar es Salaam, Tanzania
Dr. Murray Johnson, Univ. of Puget Sound, Tacoma, Wash.
Dr. R. B. Loomis, California State Univ., Long Beach
Dr. W. P. Melaney, ARS, USDA, Albuquerque, N.M.
NAMRU-3, Cairo, A.R.E.: Dr. H. Hoogstraal and Dr. M. N. Kaiser
Mr. R. O'Donnell, University of Montana, Missoula
Dr. T. A. Okereke, University of Benin, Nigeria
Pacific Research Station (NIAID), Honolulu, Hawaii: Dr. Leon Rosen and Dr. D. Gubler
Dr. Richard M. Poché, Environmental Sciences Division, Stearns-Roger, Inc., Denver
Mr. James Reddell, Texas Tech University, Lubbock
Dr. Jon P. Shoemaker, VA Hospital, Huntington, W. Va.
Dr. D. Sonenshine, Old Dominion Univ., Norfolk, Va.
Dr. R. J. Tatchell, Improvement of Tick Control Project, c/o UNDP, Dar es Salaam, Tanzania

Cooperating Units: (cont'd)

Miss Jane B. Walker, Div. of Vet. Services,
Onderstepoort, Republic of South Africa
Mr. James Webb, Texas Tech University, Lubbock
Dr. D. E. Worley, Vet. Res. Lab., Montana State Univ.,
Bozeman

Man Years:

Total:	4
Professional:	1 1/12
Other:	2 11/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
PL-480 03-036-N	C. M. Clifford J. E. Keirans	NAMRU-3 Cairo, Egypt	H. Hoogstraal	$58,800

Project Description:

The primary objective of this project is the systematic treatment, biology, ecology, and colonization of parasitic arthropods of known and potential medical importance.

A new genus of tick, Nothoaspis, has just been described. This member of the Argasidae is one of only 5 known genera in the family. It is presently found in a single cave in Campeché, Mexico, and is presumed to be a parasite of insectivorous bats. Recent collections in this cave have provided the hitherto unknown nymph of this tick.

Using the technique of scanning electron microscopy, we are reviewing African species of the genus Rhipicephalus, primarily the females of this group. Descriptions of 2 new species of the genus Ixodes and hitherto unknown stages of a third are in press, using SEM photos of taxonomically important characters. The genus Ixodes plays an important role in the transmission of viruses that cause illness in man. A comprehensive review of the 33 species of the genus Ixodes known to occur in the United States is nearing completion. Included in this project are the known distribution and host records for the various species and a key to the adults, utilizing over 300 SEM photos. Presentation of the material will be in a format enabling the user to determine species within the genus Ixodes without benefit of a SEM.

One new genus and 17 new species of parasitic mites were described, all from Venezuela.

The first year of a contract is nearing completion with NIH to edit, correct, and enter tick data at RML onto paper tape and to enter these data into the SELGEM computer at the Smithsonian Institution. During this time 31,943 tick records have been entered onto paper tape. Of these, 10,453 have been forwarded to the Smithsonian computer. Thus far, we have not received any edit copies from the Smithsonian. Authorization for extension of this contract for a second year has been approved. An on-site visit to RML by a member of NAMRU-3, Cairo, is scheduled for early May. Under the auspices of PL-480 contract 03-036-N this investigator will arrange to have the voluminous tick data at NAMRU-3 entered into the computer in a format compatible with RML records.

A new PL-480 agreement entitled "Interrelationships between ticks, vertebrates (including humans), and infectious agents, especially viruses" between NAMRU-3 and NIH, DHEW, became effective in April 1975. During the final year of the old project 03-005-1, on-site visits to NAMRU-3 were made by Drs. Keirans and Clifford. This year the 5th volume of "Bibliography of ticks and tick-borne diseases" was published under funds from this agreement.

With the acquisition of a full-time insectary attendant the RML once again has the capability of providing parasitic arthropods (especially ticks) to meet the research needs of staff scientists at RML. Currently, 31 tick, 3 mite and 1 mosquito species are under colonization.

In the study of every vector-borne disease, it is essential first to identify the species under investigation and to distinguish it from others with different, but previously unrecognized, structural and biological characteristics. This is a necessary prerequisite to all subsequent research in which parasitic arthropods are involved in human disease. As research on tick-borne diseases continues, taxonomically and biologically trained personnel of this section have been increasingly requested to assist in problems of systematics, biology and vector potential of ticks and mites in most areas of the world. In addition, numerous routine identifications are given and information pertaining to protection from and control of medically important arthropods is provided.

Keyword Descriptors: ticks, mites, systematics, scanning electron microscopy, host-ectoparasite interrelationships, vectors

Publications:

Brennan, J. M. and Reed, J. T.: The genus Eutrombicula in Venezuela (Acarina: Trombiculidae). J. Parasitol. 60: 699-711, 1974.

Keirans, J. E. and Clifford, C. M.: Ixodes (Pholeoixodes) conepati Cooley and Kohls (Acarina: Ixodidae): description of the immature stages from rock squirrels in Texas. J. Med. Entomol. 11: 367-369, 1974.

Chaniotis, B. N.: Phlebotomine sandflies in Montana: first report. Mosquito News 34: 334-335, 1974.

Philip, C. B.: Tribe II. Ehrlichieae Philip 1957, 948. In Buchanan, R. E. et al. (Eds.): Bergey's Manual of Determinative Bacteriology (ed. 8). Baltimore, The Williams & Wilkins Company, 1974, pp. 893-897.

Keirans, J. E. and Clifford, C. M.: Nothoaspis reddelli, new genus and new species (Ixodoidea: Argasidae), from a bat cave in Mexico. Ann. Entomol. Soc. Am. 68: 81-85, 1975.

Saunders, R. C. and Yunker, C. E.: Venezuelan Macronyssidae. IV. A new genus and species of rodent-parasitizing Ornithonyssinae (Acarina, Macronyssidae). J. Med. Entomol. 11: 736-759, 1975.

In press:

Keirans, J. E.: A review of the phoretic relationship between Mallophaga (Phthiraptera: Insecta) and Hippoboscidae (Diptera: Insecta). J. Med. Entomol.

Hoogstraal, H., Kaiser, M. N., Clifford, C. M., and Keirans, J. E.: The subgenus Persicargas (Ixodoidea: Argasidae: Argas). 24. A. (P.) ricei, new species, a parasite of vultures in Texas. Ann. Entomol. Soc. Am.

Clifford, C. M., Hoogstraal, H., and Keirans, J. E.: The Ixodes ticks (Acarina: Ixodidae) of Nepal. J. Med. Entomol.

Keirans, J. E.: Dermacentor andersoni Stiles, 1908 (Acarina: Ixodidae): proposed validation under the Plenary Powers. Bull. Zool. Nomencl.

In press (cont'd)

Herrin, C. S. and Yunker, C. E.: Systematics of Neotropical *Hirstionyssus* mites with special emphasis on Venezuela (Acarina: Mesostigmata). *Brigham Young Univ. Sci. Bull.*

Keirans, J. E.: Records of phoretic attachment of Mallophaga (Insecta: Phthiraptera) on insects other than Hippoboscidae. *J. Med. Entomol.*

Stark, H. E., Inlao, I., Clifford, C. M., Keirans, J. E., and Lakshana, P: A preliminary checklist of the ticks of Thailand. *Appl. Sci. Res. Corp. J. Thailand*

Clifford, C. M., Theiler, G., and Baker, M.: *Ixodes* (*Afrixodes*) *drakensbergensis* n. sp. from domestic and wild animals in Natal, Republic of South Africa. *Onderstepoort J. Vet. Res.*

Clifford, C. M., Walker, J. B., and Keirans, J. E.: *Ixodes* (*Afrixodes*) *bakeri* Arthur & Clifford (Acarina: Ixodidae): description of the male and immature stages from rodents and insectivores in South Africa. *Onderstepoort J. Vet. Res.*

Poché, R. M. and Keirans, J. E.: First report of a tick *Ornithodoros rossi* (Acarina: Argasidae) from the spotted bat, *Euderma maculatum* (Chiroptera: Vespertilionidae). *J. Med. Entomol.*

Brennan, J. M. and van Bronswijk, J.E.M.H.: New records of Surinam and certain French Guiana chiggers with the description of a new species of *Loomisia* Brennan and Reed, 1972 (Acarina: Trombiculidae). *J. Parasitol.*

Brennan, J. M. and Reed, J. T.: A list of Venezuelan chiggers, particularly of small mammalian hosts (Acarina: Trombiculidae). *Brigham Young Univ. Sci. Bull.*

Reed, J. T. and Brennan, J. M.: The subfamily Leeuwenhoekinae in the Neotropics (Acarina: Trombiculidae). *Brigham Young Univ. Sci. Bull.*

Keirans, J. E.: The Rocky Mountain wood tick and the American dog tick. Chapter in *Great Plains Range and Livestock Management.*

Clifford, C. M. and Walker, J. B.: *Ixodes* (*Afrixodes*) *neitzi*, n. sp. (Acarina: Ixodidae) from the mountain reedbuck in South Africa. *Onderstepoort J. Vet. Res.*

Project No. Z01 AI 00070-10 RML
1. Rocky Mountain Laboratory
2. Allergy and Immunology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biological activities of the histamine sensitizing factor from Bordetella pertussis

Previous Serial Number: NIAID-184

Principal Investigators: R. K. Bergman and J. J. Munoz

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	2 2/12
Professional:	1
Other:	1 2/12

Project Description:

Primary goals are 1) to study the biological effects of substances from Bordetella pertussis, especially the heat labile substance (80° C for 30 minutes) commonly referred to as histamine sensitizing factor (HSF), and 2) to study the mechanism(s) by which these substances produce their biological effects.

The vascular permeability changes induced by HSF were investigated by measuring the distribution of radioactive human serum albumin in the various tissues of mice. We found that the vascular permeability in HSF-treated mice after an intramuscular injection of vasoactive amines (histamine or serotonin) was greater than that in saline controls. Intravenous administration of catecholamines was not as effective in blocking the increased permeability in the muscle, in contrast to their ability to block capillary permeability in the skin of HSF-treated animals. When catecholamines were given intracutaneously mixed with histamine or serotonin, epinephrine (α and β receptor agonist) was the most effective inhibitor of permeability while isoproterenol (β receptor agonist) was the least effective. This observation indicates that the β-adrenergic receptors may not be the main site of action of HSF.

The effect which HSF produces in the vascular bed of the mouse to enhance permeability is detectable in the thigh muscle within 2 days and lasts at least 31 days after intravenous administration of HSF.

Preliminary studies on cyclic AMP levels in mouse lymphocytes indicate that HSF-treated mice have higher quantities of the nucleotide in their lymphocytes than normal mice and that the level can be increased in both normal and HSF-treated mice by incubating cell suspensions in the presence of isoproterenol. This finding would indicate that HSF-treated mice are not firmly blocked at the β-adrenergic receptors.

Using the CFW-R mice reared in our laboratory, we tried to protect HSF-treated mice from histamine toxicity with various nucleotides (adenosine, AMP, ADP, ATP, cyclic AMP) as previously reported by Matsumura, Vaughan, and Tan (J. Allergy Clin. Immunol. 54: 191, 1974). We were unable to repeat their work in 3 separate trials.

We found that a highly purified preparation of HSF, which induced leukocytosis in mice, was also effective in producing hyperacute experimental allergic encephalomyelitis when administered with guinea pig spinal cord emulsion given intraperitoneally to Lewis rats.

These studies may lead to a better understanding of the exact mode of action of HSF as well as to an understanding of the interrelationships between the neuroendocrine system and immunological diseases.

Keyword Descriptors: Bordetella pertussis, histamine sensitizing factor, vascular permeability, catecholamines, β-adrenergic blockade, EAE, lymphocytosis, serotonin, histamine

Publications:

Munoz, J. J., Sogandares-Bernal, F., and Bergman, R. K.: Histamine sensitization of mice by Trichinella spiralis infection. Proc. Soc. Exp. Biol. Med. 147: 524-526, 1974.

In press:

Bergman, R. K. and Munoz, J. J.: Effect of Bordetella pertussis extract and vasoactive amines on vascular permeability. J. Allergy Clin. Immunol.

Project No. Z01 AI 00071-05 RML
1. Rocky Mountain Laboratory
2. Allergy and Immunology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Purification and immunological activities of the histamine sensitizing factor of *Bordetella pertussis*

Previous Serial Number: NIAID-184

Principal Investigators: J. J. Munoz and R. K. Bergman

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 2 4/12
Professional: 1 1/12
Other: 1 3/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
PL-480 05-042-N	J. Munoz	Inst. of Microbiology Cracow, Poland	Dr. W. Ptak	$18,432

Project Description:

The main purpose of this project is to chemically and physically characterize the histamine sensitizing factor (HSF) and to study its immunological properties. This year we purified HSF further by sucrose density gradient electrophoresis and have collected enough purified HSF to determine its chemical composition.

The purest HSF has been found to contain, by thin layer chromatography, at least 15 amino acids, which indicates that HSF contains a complex polypeptide as part of its molecule. No precipitin bands have been demonstrated by gel diffusion tests with HSF tested against many hyperimmune rabbit sera prepared against fractions rich in HSF. Weak neutralization of the HSF activity has, however, been observed with 2 hyperimmune sera and this indicates antigenicity of HSF.

Enzyme digestion studies have confirmed reports that pronase at high concentration destroys most of the HSF activity. Low concentrations of this enzyme, fully capable of digesting control protein solutions, have not destroyed HSF. We have also failed to destroy HSF with other proteolytic enzymes such as ficin, trypsin, and papain. Lipase also failed to destroy it. In its purest form HSF is still capable of inducing active immunity in mice, but this point requires further investigation because erratic results were obtained.

These observations are paving the way for a complete characterization of HSF and have opened up the possibility of studying its activity without the presence of other contaminating active substances from *Bordetella pertussis*.

Keyword Descriptors: *Bordetella pertussis*, histamine sensitizing factor, lymphocytosis promoting factor, antigenicity, adjuvants, mouse protective antigen, proteolytic enzymes

Publications:

Munoz, J., Lodmell, D. L., Race, R. E., Jackson, T. A., and Hadlow, W. J.: Delayed hypersensitivity in normal and Aleutian disease-affected mink. *Z. Immunitaetsforsch*. 147: 53-60, 1974.

Munoz, J. J., Race, R. E., and Hadlow, W. J.: Complement and hemolysin to sheep erythrocytes found in normal mink serum. *IRCS* 2: 1610, 1974.

Munoz, J. J. and Race, R. E.: Sensitivity of mink uterus to histamine and serotonin. *IRCS* 2: 1496, 1974.

Project No. Z01 AI 00072-04 RML
1. Rocky Mountain Laboratory
2. Allergy and Immunology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Host defenses in viral diseases

Previous Serial Number: NIAID-175

Principal Investigator: D. L. Lodmell

Other Investigators: M. E. Bloom, B. W. Chesebro, and D. L. Granger

Cooperating Units: Dr. Abner L. Notkins, Laboratory of Oral Medicine, NIDR, NIH

Man Years:

Total:	1 7/12
Professional:	10/12
Other:	9/12

Project Description:

The major objective of this project is to elucidate the immunological mechanisms of host defense against acute, persistent and latent viral infections. These studies have focused on *in vitro* assays for determining the effects of immunologically induced mediators, humoral immunity, and cell-mediated immunity on herpes simplex virus (HSV) infected cells. *In vivo* models are now being used to establish whether the mechanisms involved in *in vitro* models can be directly applicable to virus infections in the intact animal.

Additional *in vitro* studies have shown that activation by tuberculin (PPD) of sensitized leukocytes obtained from complete Freund's adjuvant-inoculated rabbits resulted in a significant reduction in HSV plaque numbers and plaque size. Furthermore, low concentrations of these leukocytes when exposed to PPD inhibited viral replication without causing toxicity to the monolayer. The interferon obtained by activation of sensitized leukocytes was shown to be highly efficient in that only one leukocyte per 200 target cells inhibited HSV replication by over 90%. We also showed that the interferon produced from activated macrophages differs in type from that produced from activated splenic lymphocytes.

Our principal *in vivo* model is vaccinia virus infections of rabbit skin. This model is ideal for these studies because 1) infected cells can be immunologically destroyed by specific antivaccinia antibody and complement, 2) the virus is susceptible to interferon, 3) it will replicate and spread (forming plaques) in the presence of high concentrations of specific antibody, and 4) high concentrations of virus can be produced and isolated from the skin. Recent experiments have shown that an antiviral mediator can be stimulated in rabbit skin by a specific immunological reaction and that this mediator can be isolated in high titers. Stimulation of the mediator at the site of a previously established vaccinia infection in the skin (4 logs) results in a marked inhibition of virus replication early in infection, and almost complete inhibition in replication, when untreated lesions on the same rabbit contain >6 logs of virus.

These stimulating and provocative studies of immunological defense against viral infections will continue to be pursued in the vaccinia model, and in a newly developed model concerning a systemic virus infection of mice.

Keyword Descriptors: herpes simplex virus, vaccinia virus, leukocytes, mediators, macrophages, interferon

Publications:

Lodmell, D. L. and Notkins, A. L.: Cellular immunity to herpes simplex virus mediated by interferon. *J. Exp. Med*. 140: 764-778, 1974.

In press:

Notkins, A. L. and Lodmell, D. L.: Cellular immune mechanisms in viral infections. *Perspectives in Virology*, 1975.

Project No. Z01 AI 00073-10 RML
1. Rocky Mountain Laboratory
2. Allergy and Immunology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanisms of immunity in mammals and lower vertebrates

Previous Serial Number: NIAID-175

Principal Investigators: J. E. Coe and J. L. Portis

Other Investigators: B. W. Chesebro, M. E. Bloom, L. A. Thomas, J. F. Bell, and W. J. Hadlow

Cooperating Units: Dr. Ira Green, NIAID, NIH, Bethesda, Md.
Dr. Terry Jackson, USDA, Ames, Iowa

Man Years:

Total:	3 6/12
Professional:	1 8/12
Other:	1 10/12

Project Description:

Our studies have centered primarily around the immune systems of the Syrian hamster with the objective of utilizing this information for studies on host resistance to tumors and infectious agents. Cytophilic antibody has been demonstrated in other species but is unknown in the Syrian hamster. We found that the hamster macrophage has a membrane receptor that binds both $7S\gamma_1$ and $7S\gamma_2$ although the receptor manifests a 20-fold greater specificity for $7S\gamma_1$. Unlike cytophilic immunoglobulins in other species, $7S\gamma_1$ antibody did not enhance phagocytosis of the antigen or particle with which it combined whereas $7S\gamma_2$ behaved like other opsonins and facilitated ingestion by macrophages. These unique properties of $7S\gamma_1$ suggest a more specialized function for this Ig class, for instance, the binding of antigen to macrophages with subsequent induction of antibody formation.

Our studies indicate that cytophilic immunoglobulins of many mammals share common Fc determinants. Of the mammalian Ig tested only 2—bovine and guinea pig—lacked cytophilic activity for hamster macrophages. Guinea pig γ_2 but not γ_1 was cytophilic for homologous macrophages, but lacked cytophilic activity for heterologous macrophages. In this respect, guinea pig γ_2 resembled hamster γ_1 for the latter also bound only to homologous macrophages. Thus, guinea pig macrophages had a receptor specific for a determinant on homologous $7S\gamma_2$ and hamster macrophages had a receptor specific for a configuration on homologous $7S\gamma_1$. Yet it was found that hamster $7S\gamma_2$ was cytophilic for both hamster and guinea pig macrophages. This suggests that hamster γ_2 has at least 2 different cytophilic determinants. Further work will determine if nonphagocytic or phagocytic cytophilic antibody is most effective in producing immunity.

We have shown before that the types of Ig produced depend on the form of the immunizing antigen. For instance, the Ig response to rabies virus is limited to $7S\gamma_2$. On the other hand, hamsters immunized with HEA in complete Freund's adjuvant (CFA) make antibody in the IgM, $7S\gamma_1$ and $7S\gamma_2$ classes, but those immunized with HEA in saline produce primarily a $7S\gamma_1$ antibody and do not respond in the $7S\gamma_2$ class, when challenged with HEA in CFA. Using the Cunningham modification of the Jerne plaque assay, the above observations have been confirmed and quantitated. When irradiated hamsters that had received lymphoid cells from animals primed with HEA in saline were challenged with HEA in CFA, an anamnestic $7S\gamma_1$ response was seen, but the $7S\gamma_2$ response was suppressed 200-fold for at least 21 days. Likewise, it appeared that the IgM antibody response was also depressed. Because of recent observations on suppressive effects of subpopulations of T cells, we investigated the possible role of active cell-mediated suppression in this experimental model. Preliminary experiments suggest the existence of a suppressor population of cells in the thymus of animals primed with HEA in saline. Furthermore, this suppressive effect was detected after administration of antigen in drinking water, which suggests that this selective induction-suppression phenomenon could exist also in nature. Other experiments in progress indicate the presence of an inhibitor in spleen which prevents the maturation of antibody forming cells in that organ _in vivo._

A protein has been detected in Syrian hamsters which is only transiently detected in males (peak on day 21) but is consistently found in young and old female hamsters (usually around 1.5 mg/ml serum). The hormonal control of the synthesis of this sex-linked protein, called female protein (FP), has been shown by castration and hormone administration. FP appeared in castrated hamsters and it rapidly decreased when these hamsters were treated with testosterone and reappeared upon cessation of hormone treatments. Diethylstilbesterol by itself could induce comparable synthesis of FP. FP in neonatal male hamsters is not derived via the placenta nor absorbed from the milk. FP is an interesting model of hormonally controlled protein synthesis and the function of this protein is unknown. However, FP appears in adult male hamsters occasionally after inoculation with various antigens and frequently in male tumor bearing hamsters. Future work will determine if FP is responsible for the reported sex differences in hamster immunity.

In collaboration with Dr. Leo Thomas, we have further characterized the Ig system in garter snakes. Two low molecular weight (8 to 9S) Ig are now apparent. One of them, Ig1, is slightly faster electrophoretically and slightly heavier by sucrose density gradient analysis than Ig2. Furthermore, Ig1, present at very low levels in normal snake serum, becomes quite prominent after immunization with protein antigen and actually seems to contain most of the anti-protein antibody. In fact, antisera specific for this Ig1 have been achieved utilizing absorption with normal whole snake serum. Serum macroglobulin has an S value around 21, is produced in a transient antibody response to protein antigen, but appears mainly to function as the secretory Ig. IgM is the only Ig in bile and the predominant Ig in *succus enterieus* where the IgM:IgG concentration ratio is more than 50-fold that of serum. However, the IgMs in these fluids are not completely identical because of S value differences (serum IgM = 21S, *succus* IgM = 21 and 15S, bile IgM = 17S) and antigenic differences detectable as spurring on gel diffusion assay with rabbit specific anti-bile IgM antisera. Now, for the first time, garter snake antibodies to WEE can be detected easily by a 2-step precipitation assay. The existence of specific anti-Ig sera will clarify the ontology of antibody response in snakes and thereby serve as a useful epidemiological tool.

Other on-going projects in tumor immunity and Aleutian disease etiology are being conducted in collaboration with Drs. Chesebro and Bloom. Our laboratory also functions as a source of information and material for other scientists at the RML and University of Montana.

Keyword Descriptors: Syrian hamster immunology, cytophilic antibody, immunological tolerance, sex-linked protein

Publications:

Coe, J. E. and Green, I.: Brief communication: B-cell origin of hamster lymphoid tumors induced by simian virus 40. *J. Natl. Cancer Inst.* 54: 269-270, 1975.

Project No. Z01 AI 00074-03 RML
1. Rocky Mountain Laboratory
2. Allergy and Immunology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Host defense mechanisms in chronic viral and neoplastic diseases

Previous Serial Number: NIAID-175

Principal Investigators: B. W. Chesebro and M. E. Bloom

Other Investigators: J. E. Coe, W. J. Hadlow, T. A. Jackson, D. L. Lodmell, J. L. Portis, and R. E. Race

Cooperating Units: Dr. Jack Stimpfling, McLaughlin Research Institute, Great Falls, Montana

Man Years:

Total:	4 4/12
Professional:	1 10/12
Other:	2 6/12

Project Description:

Friend mouse leukemia virus. The major goal of this project is elucidation of host defense mechanisms involved in recovery from virus induced leukemia in mice. A single gene (RFV-1) which influences the ability of mice to recover from leukemia has been characterized and found to map in the D-region of the major histocompatibility locus (H-2). To study possible mechanisms of action of the RFV-1 gene, we investigated the humoral and cellular components of the host immune response. Neutralizing anti-viral antibodies and cytotoxic anti-leukemia cell antibodies appear about 2 weeks after viral inoculation and coincide with disappearance of infectious virus from the blood and spleen; however, presence of these antibodies does not correlate with recovery from leukemia. Recovery does correlate with appearance of specific anti-leukemia cell cytotoxic lymphocytes in the spleens of recovered mice. These cytotoxic cells have been shown to be T lymphocytes which function *in vitro* independently of macrophages and B lymphocytes. The RFV-1 gene does not appear to exert its influence on recovery via an absolute control over appearance of these cytotoxic lymphocytes. It remains to be determined whether these cytotoxic cells are the major effectors of recovery from leukemia or merely appear in a secondary fashion following recovery, which could be mediated by an altogether different mechanism.

In addition, the interactions of normal H-2 and I region associated cell surface antigens with viral induced antigens are being studied with particular reference to the possible role of the RFV-1 gene in influencing recognition and elimination of leukemia cells by the host immune system.

Aleutian disease virus (ADV) of mink. Study on ADV of mink has involved the infectious virus as well as the host immune response. The goal has been to determine the relative roles of these two factors in both infection and pathogenesis of clinical immune complex disease.

The Utah-I strain of ADV has been purified by CsCl density gradient centrifugation and banded at a density of 1.36 gm/ml. Twenty-five nm virus particles were seen with the electron microscope. Based on density and morphology, ADV is probably a parvovirus. Using a feline kidney cell line (CRFK), we have quantitatively assayed ADV *in vitro*, using an immunofluorescence technique.

The course of anti-ADV antibody response of sapphire and pastel mink to the Pullman strain of ADV has been studied with CIEP. Both sapphires and pastels mount a vigorous initial anti-viral antibody response, but whereas sapphires develop chronic viremia, sustained high antibody levels, and fatal immune complex disease, pastels have a transient high antibody response that subsequently falls to lower levels, and they do not develop disease. Based on preliminary data from the slow virus unit at RML (Hadlow), these differences in antibody response appear to reflect the patterns of viral replication which, together with antibody, determine the induction and outcome of immune complex disease in the 2 mink strains.

Study of the mitogen induced proliferative response of lymphoid cells from normal and ADV-infected pastel mink has shown that infected animals have depressed response to both T- and B-cell mitogens, and has also suggested the existence of 2 differing T-cell populations.

Keyword Descriptors: Friend mouse leukemia virus, Aleutian disease virus, host immune defenses

Publications:

Chesebro, B., Wehrly, K., and Stimpfling, J.: Host genetic control of recovery from Friend leukemia virus induced splenomegaly: mapping of a gene within the major histocompatibility complex. *J. Exp. Med.* 140: 1457-1467, 1974.

Chesebro, B., Bloom, M., Hadlow, W., and Race, R.: Purification and ultrastructure of Aleutian disease virus of mink. Nature 254: 456-457, 1975.

In press:

Bloom, M. and Jackson, T. A.: Virus-like particles in buffy coat cells of normal goats and goats infected with progressive pneumonia virus. Am. J. Vet. Res.

Project No. ZO1 AI 00075-01 RML
1. Rocky Mountain Laboratory
2. Allergy and Immunology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Immunology of pathogenic fungi

Previous Serial Number: None

Principal Investigator: H. F. Hasenclever

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

The serologic relationships among pathogenic and nonpathogenic yeasts and the immune response of experimental animals to *Candida albicans* are the major areas for investigation.

Previous work had shown some of the interrelatedness among the serotypes of *Saccharomyces telluris* and the antigenic groups of *C. albicans*. Recent results have shown that the comparative antigenic structures of these yeasts can be illustrated as follows: *S. telluris* A-AB(st), *S. telluris* B-B(st), *S. telluris* C-(st), *C. albicans* A-AB(ca), and *C. albicans* B-B(ca). Therefore, antigen(st) is common only to the 3 serotypes of *S. telluris*, and (ca) is found only in the 2 groups of *C. albicans*. Antigens A and B are found as indicated.

Studies on antibody synthesis in rabbits inoculated daily with 1×10^8 heat-killed *C. albicans* A cells intravenously show that the response to the antigen common to both groups occurs after 8-11 days and that the Group A reactivity does not appear until the 15th day. Survivors from a single dose of 1×10^6 viable cells intravenously apparently do not receive sufficient immunogenic stimulation to respond to the Group A specificity.

This work characterizes the immune response to *C. albicans* and the antigenic relationships among yeasts. Information obtained may help elucidate problems associated with diagnosis and management of fungal disease.

Keyword Descriptors: *Candida albicans*, *Saccharomyces telluris*, antigens, serotypes, antibodies, groups, yeasts, fungi

Publications:

Kocan, R. M. and Hasenclever, H. F.: Seasonal variations of the upper digestive tract yeast flora of feral pigeons. *J. Wildl. Dis.* 10: 263-266, 1974.

Reiss, E., Stone, S. H., and Hasenclever, H. F.: Serological and cellular immune activity of peptidoglucomannan fractions of *Candida albicans* cell walls. *Infect. Immun.* 9: 881-890, 1974.

Reiss, E., Mitchell, W. O., Stone, S. H., and Hasenclever, H. F.: Cellular immune activity of a galactomannan-protein complex from mycelia of *Histoplasma capsulatum*. *Infect. Immun.* 10: 802-809, 1974.

Hasenclever, H. F. and Piggott, W. R.: Colonization of soil by *Histoplasma capsulatum*: I. Factors affecting its continuity in a given site. *Health Lab. Sci.* 11: 197-200, 1974.

Hasenclever, H. F. and Kocan, R. M.: *Candida albicans* associated with the gastrointestinal tract of the common pigeon (*Columba livia*). *Sabouraudia* 13: 116-120, 1975.

Project No. Z01 AI 00076-17 RML
1. Rocky Mountain Laboratory
2. Molecular Biology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanisms of immunopotentiation by mycobacterial fractions

Previous Serial Number: NIAID-177

Principal Investigators: E. Ribi, D. L. Granger, K. Yamamoto (Visiting Scientist), and M. T. Kelly

Other Investigators: R. F. Smith

Cooperating Units: Dr. I. Azuma, Osaka University, Osaka, Japan
Dr. W. Brehmer, Robert Koch Institute, Berlin, Germany
Hamilton Biochemical Research Lab.: Dr. R. Parker and Mr. S. M. Strain

Man Years:

Total:	7 11/12
Professional:	2 9/12
Other:	5 2/12

Project Description:

We have reported that BCG vaccines protect mice against pulmonary tuberculosis when challenged with aerosols containing a few virulent tubercle bacilli 4 weeks after intravenous immunization. The number of virulent bacilli in the lungs 4 weeks after infection was used as the measure of protection. In this test system, BCG cell walls (CW) attached to oil droplets and suspended in saline were as effective as viable BCG vaccines; their efficacy paralleled the extent of granuloma produced in the lung tissue, as estimated by lung weight increase.

The complex nature of CW and whole cells made it impossible to separate protection from granuloma formation; therefore, materials were further fractionated to determine whether individual components or chemical constituents could be correlated with particular effects and, if possible, to define the minimal composition of a protective vaccine. We reported last year that a high degree of protection could be achieved by an oil-treated vaccine composed of tubercle protein (PPD) and P3, a nonantigenic, nonsensitizing, high molecular weight trehalose ester of mycolic acids isolated from *Mycobacterium tuberculosis*.

We now report that proteins from tubercle bacilli could be replaced by antigenically nonrelated proteins such as bovine serum albumin (BSA). With this simplest of vaccines the degree of pulmonary granuloma also paralleled the suppression of bacterial growth (protection). Neither P3, PPD, nor BSA does so alone. This suggests that the protein may be the antigen to which the host becomes immunized through the adjuvant action of P3. The effects are expressed pathologically as an intense granulomatous response and immunologically as resistance (nonspecific in the case of P3 + BSA) against aerosol infection with tubercle bacilli. The degree of protection depended primarily on the extent of pulmonary granuloma present at the time of challenge. Thus, a combination of P3 + acetylated bovine serum albumin (Ac-BSA) produced an extensive granulomatous response that was maximal 2 weeks after immunization and after 4 weeks had declined about 50%. The granuloma produced by the slower acting BCG-CW at 4 weeks was about the same as that induced by P3 + Ac-BSA at 2 weeks. Accordingly, mice immunized with P3 + Ac-BSA and challenged 2 weeks after immunization, were as well-protected as those vaccinated with BCG-CW and infected 4 weeks later. The granuloma and concomitant ability to resist infection induced by BCG-CW, although slower to develop, persisted over a much longer period of time. On the other hand, the granuloma and protection induced by Ac-BSA + P3 developed more rapidly but did not persist.

Also studied were the effects on pulmonary granuloma formation and protection in BCG-CW-immunized mice of administration of various substances, such as specific antigens or nonspecific agents (silica, Con A, or carrageenan) which are considered to have influence on lymphocytes or macrophages involved in cell-mediated immunity. Results of desensitization experiments showed that multiple intravenous injections of BCG protoplasm (specific antigen) inhibited the footpad delayed hypersensitivity, macrophage migration inhibitory activity of lung cells and pulmonary granuloma formation, as well as resistance against aerosol challenge with H37Rv. Treatment with Con A, silica, or carrageenan induced only abrogation of footpad delayed hypersensitivity but did not prevent pulmonary granuloma formation.

The results presented here, considered in the light of recent publications from this and other laboratories, are thought to dictate rather firm conclusions regarding interrelationships between pulmonary granuloma, delayed-type hypersensitivity, cell-mediated immunity and protection in the mouse model system under study.

Since P3, in combination with other microbial fractions and oil droplets, also was capable of causing regression of guinea pig hepatoma (see Project No. Z01 AI 00078-03 RML), its function, in combination with purified, defined protein antigens, was studied in simplified *in vivo* and *in vitro* models.

Intradermal injection of guinea pigs with BSA + P3-oil droplet mixture induced DH to BSA. This was attended by chronic inflammation at the site of injection and in the first draining node characterized histologically as granulomatous reaction. A method for separating oil droplets from the aqueous phase of this mixture showed that in the presence of P3 a significant amount of BSA became irreversibly associated with oil droplets and that these oil droplets were immunogenic. The P3-mediated association occurred only when BSA and P3 were mixed in mineral oil prior to emulsification and was shown to depend on the concentration P3, antigen (BSA) and oil in a linear fashion. When fluorescein conjugated antigen + P3 oil droplet emulsion was prepared, one could visualize fluorescent particles of protein in or on oil droplets. This effect was not seen in the absence of P3. Results of these studies demonstrate that one important effect of P3 is to associate antigen with oil droplets when the three are mixed together prior to emulsification. This phenomenon appears to be a necessary event for induction of DH and granulomatous reaction in this system. It is suggested that P3 may exert a similar action with bacterial fractions possessing antitumor effects.

Macrophages play an important role in antituberculous immunity, and histological studies suggest that they may be of central importance in tumor regression mediated by BCG. Macrophages from normal animals lack significant antibacterial and antitumor activities, and such macrophages must be "activated" in order to carry out these functions. Therefore, one of the effects of the immunopotentiating mycobacterial components might be to induce macrophage activation.

The ability of peritoneal macrophages to kill Listeria monocytogenes in vitro was used to assess macrophage activation. Guinea pigs vaccinated intraperitoneally with BCG cell walls associated with oil droplets developed macrophages with potent listericidal activity. These studies suggest that macrophage activation may be one important aspect of the immunopotentiating effects of nonviable mycobacterial components.

Keyword Descriptors: tuberculosis, immunization against tuberculosis, BCG, nonliving vaccines, mycobacterial fractions, trehalose mycolates, immunopotentiation, cell-mediated immunity, delayed type hypersensitivity, macrophage activation

Publications:

Meyer, T. J., Anacker, R. L., and Ribi, E.: Effects of pretreatment of mice with BCG cell walls in saline on subsequent vaccination with BCG oil droplet vaccine. *Cell. Immunol*. 14: 52-65, 1974.

Azuma, I., Yamamura, Y., and Ribi, E.: Preparation of adjuvant-active, tuberculin-negative peptidoglycolipid from human tubercle bacilli. *Jap. J. Microbiol*. 18: 327-332, 1974.

Meyer, T. J., Azuma, I., and Ribi, E. E.: Biologically active components from mycobacterial cell walls. III. Production of experimental allergic encephalomyelitis in guinea pigs. *Immunology* 28: 219-229, 1974.

Ribi, E., Meyer, T. J., Azuma, I., Parker, R., and Brehmer, W.: Biologically active components from mycobacterial cell walls. IV. Protection of mice against aerosol infection with virulent *Mycobacterium tuberculosis*. *Cell. Immunol*. 16: 1-10, 1975.

Meyer, T. J., Ribi, E., and Azuma, I.: Biologically active components from mycobacterial cell walls. V. Granuloma formation in mouse lungs and guinea pig skin. *Cell. Immunol*. 16: 11-24, 1975.

Salvin, S. B., Ribi, E., Granger, D. L., and Youngner, J. S.: Migration inhibitory factor and Type II interferon in the circulation of mice sensitized with mycobacterial components. *J. Immunol*. 114: 354-359, 1975.

Ribi, E., Azuma, I., Meyer, T., Parker, R., Brehmer, W., and Yamamura, Y.: Chemical structure and biological functions of mycobacterial cell walls with special reference to immunity against airborne infection with *M. tuberculosis* strain H37Rv. *Bull. Int. Union Tuberc*. 49: 340-350, 1974.

Project No. Z01 AI 00077-19 RML
1. Rocky Mountain Laboratory
2. Molecular Biology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Structure and biologic activity of endotoxins

Previous Serial Number: NIAID-178

Principal Investigator: K. C. Milner

Other Investigators: D. L. Granger, E. Ribi, J. E. Cutler (NIH Postdoctoral Fellow)

Cooperating Units: Dr. T. Hofstad, University of Bergen, Norway
Dr. J. A. Rudbach, University of Montana, Missoula
Dr. J. R. Vestal, University of Cincinnati, Ohio

Man Years:

Total:	2 8/12
Professional:	10/12
Other:	1 10/12

Project Description:

This project has developed from the central purpose of elucidating the structural basis for host-reactive properties of bacterial endotoxins. Capabilities for physicochemical analysis and biological assay acquired in these studies have been applied to other projects of the RML Section of Molecular Biology and of cooperating units.

Currently, most effort is directed toward research on tuberculosis and cancer immunotherapy where we found that endotoxin can be effectively substituted for tuberculoprotein in oil-droplet vaccines that protect mice against tuberculosis and regress malignant hepatomas in guinea pigs. In part, this stemmed from our discovery that the most active preparation of PPD was liberally contaminated with endotoxin. There has been a long, embarrassing history of effects ascribed to different biological materials that were later found to be occasioned by contaminating endotoxin, and there is every reason to believe that only the tip of this "iceberg" has come to light. It should be assumed that all experimental biological preparations contain endotoxin, unless sensitive bioassays have proved otherwise; and it should be much more frequently determined if endotoxin will duplicate effects that are being attributed to something else.

Brief mention of some continuing aspects of this project follow. 1) It has been found that oil droplet vaccines with P3 (trehalose mycolate) and specific lipopolysaccharide (LPS) will protect mice against challenge with virulent *Salmonella typhimurium* better than LPS alone or killed whole cells. The objective is to achieve, with nonviable immunogens, the solid type of resistance that is now obtained only with attenuated live vaccines. We have also shown that oiled BCG cell-wall vaccines sensitize mice to endotoxin to the same extent as viable BCG. 2) With Prof. Tor Hofstad, we have shown that several oral anaerobic bacteria produce endotoxins. There is evidence that these are important contributors to the pathogenesis of periodontal disease. 3) With Dr. Vestal, we are studying endotoxic extracts from *Acinetobacter calcoaceticus* (*Mima polymorpha*). These normally free-living or saprophytic bacteria are being increasingly implicated in nosocomial infections. 4) Work on the limulus amebocyte lysate test has been temporarily suspended, for lack of lysate and essential personnel. A manuscript submitted to Dr. D. Gröschel for inclusion in a monograph was recently returned because too many prospective contributors had withdrawn from the project.

Dr. Cutler left in late summer to accept regular appointment as Assistant Professor at Montana State University, Bozeman.

Keyword Descriptors: endotoxin, lipopolysaccharide, *Acinetobacter*, periodontoclasia, salmonellosis, tumor therapy, tuberculosis, immunization to tuberculoprotein

Publications:

Cutler, J. E.: A simple *in vitro* method for studies on chemotaxis. *Proc. Soc. Exp. Biol. Med.* 147: 471-474, 1974.

Project No. Z01 AI 00078-03 RML
1. Rocky Mountain Laboratory
2. Molecular Biology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Microbial components in cancer immunotherapy

Previous Serial Number: NIAID-179

Principal Investigators: E. Ribi and D. L. Granger

Other Investigators: M. T. Kelly, K. C. Milner, R. F. Smith, and K. Yamamoto (Visiting Scientist)

Cooperating Units: Biology Branch, NCI, NIH: Dr. B. Zbar and Dr. H. J. Rapp
Dr. W. Brehmer, Robert Koch Institute, Berlin, Germany
Dr. J. Gutterman, M. D. Anderson Hospital and Tumor Institute, University of Texas, Houston
Hamilton Biochemical Research Lab.: Dr. R. Parker and Mr. S. M. Strain
Osaka University, Osaka, Japan: Dr. I. Azuma and Dr. Y. Yamamura

Man Years:

Total:	6 8/12
Professional:	2 11/12
Other:	3 9/12

Project Description:

Viable BCG vaccines are useful in therapy of neoplasms in experimental animals, and clinical evaluation of BCG for the treatment of cancer is under way in at least 50 centers throughout the world. Using the syngeneic guinea pig line-10 hepatoma immunotherapy model, we have shown that BCG cell walls (CW) associated with tiny oil droplets are as effective as viable BCG in regressing established tumors and metastases after tumors were injected with these preparations (cure rates of about 60%).

There are both practical and theoretical advantages for using nonliving preparations for cancer immunotherapy. The risk of progressive BCG infection in immunosuppressed patients could be obviated, and the use of nonviable materials eliminates the necessity for preserving living cells. Nonliving fractions can be standardized and shipped and stored in the dry state without loss of potency. We report here that 300 μg of CW and CW skeleton preparations from Mycobacterium phlei, a fast growing saprophyte of group IV atypical mycobacteria, regressed tumors in 50 to 60% of the animals. Because M. phlei grows more rapidly, it can be advantageously substituted for BCG to prepare stable, nonliving immunologic adjuvants of defined composition and consistently high potency. Large quantities can be harvested from 2-day-old cultures whereas it requires 2 weeks to grow BCG. This long cultivation period has been suspected to be one reason for the variable potency encountered in BCG vaccines.

One of the most essential fractions in tumor immunotherapy is P3, a nonsensitizing, nonantigenic trehalose mycolate, related to, but not identical with, cord factor. The addition of 300 μg of P3 isolated from M. tuberculosis strain Aoyama B to 300 μg of CW from either BCG or M. phlei significantly increased their tumor regressive potency, a 90% cure rate. Oil droplet preparations containing P3 and certain bacterial endotoxins also cured up to 90% in the same tumor system. Furthermore, endotoxin P3 complex regressed tumors faster, and older, more highly metastasized tumors could be successfully treated. These results are fundamentally different from earlier findings on the tumor damaging effects of endotoxin. Endotoxin alone produces a hemorrhagic, Shwartzman-like response in malignant tissue leading to significant, but usually only temporary, tumor regression. The most highly lethal and pyrogenic endotoxins from wild-type bacteria were not very effective in our tumor system. Best results were obtained with endotoxins from rough (Re) mutants. Our results support the conclusion that regression of tumors depends upon the rapid development of cell-mediated immunity, augmented by P3, and directed first against a nitrogenous component of the endotoxin and later against the tumor itself.

These results demonstrate that characterization at the molecular level of bacillary components responsible for immunotherapeutic effects provides a useful method for studying the mechanisms involved in BCG-mediated tumor regression. Moreover, the use of well-defined bacterial fractions will enable us to channel the immune response into cell-mediated tumoricidal mechanisms and eliminate possible tumor-enhancing effects of humoral antibody, once these phenomena are clearly understood.

Japanese collaborators reported by personal communication the results of a first clinical trial of BCG cell wall-oil droplet preparations. An advanced case of melanoma was successfully treated with multiple intralesional inoculation of one of these products. Clinical trials with these materials are scheduled in the M. D. Anderson Tumor Institute, Houston, Texas, during 1975.

Project No. Z01 AI 00078-03 RML

Keyword Descriptors: cancer immunotherapy, immunopotentiation, tumor model in guinea pigs, adjuvants, mycobacterial cell wall fractions, endotoxins

Publications:

Ribi, E., Parker, R., and Milner, K.: Microparticulate gel chromatography accelerated by centrifugal force and pressure. In Glick, D. (Ed.): Methods of Biochemical Analysis. New York, John Wiley & Sons, Inc., 1974, vol. 22, pp. 355-400.

Azuma, I., Ohuchida, A., Taniyama, T., Yamamura, Y., Shoji, K., Hori, N., Tanaka, Y., and Ribi, E.: The mycolic acids of Mycobacterium rhodochorous and Nocardia corallina. Biken J. 17: 1-9, 1974.

Zbar, B., Ribi, E., Meyer, T., Azuma, I., and Rapp, H. J.: Immunotherapy of cancer: regression of established intradermal tumors after intralesional injection of mycobacterial cell walls attached to oil droplets. J. Natl. Cancer Inst. 52: 1571-1577, 1974.

Yamamura, Y., Azuma, I., Taniymama, T., Ribi, E., and Zbar, B.: Suppression of tumor growth and regression of established tumor with oil-attached mycobacterial fractions. Gann 65: 179-181, 1974.

In press:

Granger, D. L., Ribi, E., Azuma, I., Parker, R., Strain, S. M., and Smith, R. F.: Cancer immunotherapy with nonliving mycobacterial components. Proc. Chicago Symposium, 1974

Gray, G. R., Ribi, E., Granger, D., Parker, R., Azuma, I., and Yamamoto, K.: Immunotherapy of cancer: tumor suppression and regression by cell walls of Mycobacterium phlei attached to oil droplets. J. Natl. Cancer Inst.

Project No. Z01 AI 00079-16 RML
1. Rocky Mountain Laboratory
2. Epidemiology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The encephalitides

Previous Serial Number: NIAID-180

Principal Investigator: L. A. Thomas

Other Investigators: J. E. Coe

Cooperating Units: Dr. David B. Lackman, Microbiology Laboratory, Montana State Dept. of Health, Helena
Dr. Martin Skinner, Montana State Epidemiologist, Helena

Man Years:

Total:	1
Professional:	8/12
Other:	4/12

Project Description:

Investigations were continued to determine the role garter snakes might play in the ecology of Western equine encephalitis (WEE) virus, to identify field isolates from mosquitoes and ticks, and to monitor arbovirus activity in human and animal populations in the Northwest. Modification of the neutralization test by the addition of garter snake anti-gamma globulin to the serum-virus mixture has made it possible to separate snake antibody-virus complex by centrifugation. In the past, all attempts to demonstrate antibody in snakes have been negative, using the standard neutralization test. Some snakes inoculated with varied doses of virus were shown to be positive for antibody at least 528 days (experiment in progress). None of the uninoculated snakes were positive for antibody. All viremic snakes developed antibody, as did some that were not viremic. Route of inoculation (IC or SC) did not alter the viremia or antibody pattern. The findings to date are considered highly significant in studying possible overwintering mechanisms of WEE virus. Future work will consist of determining persistence of antibody in experimentally infected snakes, determining the serum fraction which is antibody-positive by employing specific anti-gamma globulin fractions in the test, and most important of all, whether garter snakes are naturally infected with WEE virus and thus might play a significant role in the ecology of WEE.

A survey of approximately 2,000 sera collected by the Red Cross from various locations in Montana were tested for hemagglutination-inhibition (HI) antibodies against WEE and St. Louis encephalitis (SLE) viruses with 100 positive (titer of 1:10 or greater) for WEE and 31 positive for SLE. Twenty human sera from Montana were submitted for encephalitis study—all were negative for complement-fixation (CF) and HI antibodies. Of 27 single horse sera, 7 were positive for WEE in both tests, 3 by HI only and one by CF only. Paired sera from 2 horses were WEE positive. Fifty sentinel chickens from the Glasgow area were bled in July and August—all were negative for HI antibodies. No mosquitoes were received for testing.

Sera were received from Nebraska, Wyoming, South Dakota, Oregon, and Washington. WEE antibodies were demonstrated in one of 2 patients from Oregon and 3 of 4 human sera and 6 of 8 horse sera from Washington. A total of 105 sera from the Alaska National Guard (collected in 1955 and 1960) were tested and found negative for HI antibodies against WEE, SLE, Tyuleniy, and Japanese B encephalitis viruses.

Of particular interest was the detection of a serological response of an RML employee against all antigens prepared from mouse brain tissue and not from yolk sac tissue. This response is the same as observed when testing patients with lupus disease. This observation appears highly significant and further study of this person to detect early evidence of a possible collagen disease is recommended.

The laboratory will continue to be of service to local and out-of-state physicians and health departments in the serological testing of sera for antibody against selected agents. It will also continue to obtain, produce, and test reagents for serological testing as well as employ the best methods in testing. Both mosquito and tick-borne viruses are under study.

Keyword Descriptors: encephalitis, arboviruses, mosquito-borne, antibody, snakes

Publications: None

Project No. Z01 AI 00080-08 RML
1. Rocky Mountain Laboratory
2. Medical Zoology and Zoonotic Diseases
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Tick-borne viral disease agents

Previous Serial Number: NIAID-181

Principal Investigators: C. M. Clifford and C. E. Yunker

Other Investigators: J. E. Keirans, R. N. Philip, and L. A. Thomas

Cooperating Units: Dr. J. Casals, Yale Arbovirus Research Unit, New Haven
Dr. E. Easton, University of Dar es Salaam, Tanzania
Dr. J. E. George, Texas Tech University, Lubbock
Mr. R. Gresbrink, Oregon State Board of Hlth., Portland
Dr. Frederick A. Murphy, CDC, Atlanta, Ga.
NAMRU-3, Cairo, Egypt: Dr. Harry Hoogstraal and Dr. James Converse
Mr. Robin Rice, University of Texas, Austin
Mrs. Margaret Sweeney, Idaho State Dept. of Hlth., Boise
Dr. Gerald Walton, University of Cork, Ireland

Man Years:

Total:	5 1/12
Professional:	1 8/12
Other:	3 5/12

Project Description:

We have attempted to determine the incidence, distribution, and ecology of tick-borne diseases that influence man and domestic animals in the United States. Of equal importance is the investigation of the vector relationships of the increasing number of viruses known or thought to be associated with ticks that may cause human illness.

Viruses in ixodid ticks. One hundred eighty-two pools of hard ticks from various localities in the U.S. and from Central Pacific islands were tested for virus in suckling mice Vero cells. Five isolations were made: all were from *Ixodes uriae* in Oregon and, with one exception, have been identified as previously recognized agents of the Kemerovo, Sakhalin and Uukuniemi groups. The exception, "28" virus, was isolated and subsequently reisolated from a single engorged nymphal tick and is apparently unique for I. uriae. Further studies on the identity of this agent are in progress.

Work was continued on the characterization of viruses collected and reported by us previously: Tillamook (Sakhalin group, previously called strain "86") and strain "38" (Uukuniemi group, near Zaliv Terpeniya), both from Oregon; Poovoot (strain "71") and Kenai (strain "1629"), both Kemerovo group agents from Alaska; and Kachemak Bay virus (strain "1628"), an Alaskan member of the Sakhalin group. Each of these are serologically distinct viruses of the seabird parasites I. uriae or I. signatus.

One hundred eleven pools of seabird (murre and gull) tissues, including blood samples obtained from embryonated eggs, were tested for the presence of these viruses with negative results. Previous studies have indicated that a high proportion of murres are immune to one or more of the above agents, and that immunity may be transferred to unhatched chicks, which may account for the lack of isolations.

We continued to investigate the possibility that these viruses were capable of being transmitted through a mosquito-vector and thus of potential epidemic significance. Tyuleniy (Three-Arch strain) virus was inoculated intrathoracically into the mosquitoes Aedes albopictus and Culiseta incidens, which were then assayed at intervals for virus. Virus was rapidly inactivated in these hosts, implying that mosquitoes are not involved in Tyuleniy virus transmission.

Last year we reported the presence of hemagglutination inhibition (HI) antibodies to Tyuleniy virus in a high percentage of common murres from Yaquina Head, Oregon. Among the positive samples were sera from newly hatched murres, implying transfer of maternal antibodies. This year we returned to Yaquina Head and obtained samples from additional birds, including a series of 16 bloods from unhatched, embryonated murre eggs. Two of the latter showed significant HI reactions to Tyuleniy virus and 6 showed low to high neutralizing titers of antibodies to Yaquina Head virus. It appears likely that maternal antibodies to these tick-borne viruses are transferred in the egg, a situation known for one other arbovirus, Russian spring-summer encephalitis, also a tick-borne agent. Additional evidence that a high percentage of murres possess antibodies to Tyuleniy virus was seen again this year. Also, 6 of 9 gull chicks had HI antibodies to Tyuleniy virus (last year a small series of gull bloods were found negative for these antibodies). Neutralizing antibodies to Yaquina Head virus were demonstrated in a high proportion of young murres, but not in gull chicks nesting in close proximity.

Rickettsia in ixodid ticks. For several years we have processed ticks from the Oregon State Health Department in an attempt to isolate Powassan virus. Although we have not been able to isolate Powassan virus, we have produced some interesting virological findings, and to date 4 strains of rickettsiae have been isolated from I. pacificus in Tillamook and Josephine counties. These strains are related to the spotted fever group of rickettsia. These results indicate that spotted fever group rickettsiae are being maintained in areas where their usual vectors (Dermacentor ticks) are not present.

Viruses in argasid ticks. Thirty pools of the bird parasite, Argas brumpti, from Tanzania were tested in suckling mice and tissue culture. Three strains isolated from tissue cultures appear to be a single type; these are poorly pathogenic in intracerebrally inoculated mice, and produce large plaques in Vero cells. Repeated CF tests with crude antigen have failed to reveal any relationship of this agent to known viruses in our collection. Additional identification tests are planned.

A unique virus reported last year from A. cooleyi, a North American parasite of migratory birds, was further characterized in preparation for registration and description as a new virus, Sunday Canyon. This virus is apparently a large, enveloped RNA virus of moderately high pathogenicity for suckling mice and Vero cells. Electron microscopic examination reveals that it is morphologically a Bunyamwera-like virus.

Further efforts were made to clarify the status of the agents isolated from Ornithodoros capensis group ticks from Manana I., off Oahu, Hawaiian Islands (see last report). Because some pools of ticks had yielded mixtures of viruses, additional ticks were tested individually and single viruses were recovered. These were identified as Johnston Atoll (Quaranfil group), Midway (Nyamanini group) and a member of the Hughes group, nearest to Soldado virus. The first two were previously known from Central Pacific islands, but this is apparently the first record of a Hughes group agent in this area.

Three strains of a Soldado-like virus have been isolated from O. (A.) maritimus collected on Saltee Island off the coast of Ireland. Soldado virus had previously been known only from O. capensis group ticks collected in Trinidad.

Assay systems for tick-borne viruses. These efforts continue because of the generally low infectivity of many tick-borne viruses for standard laboratory animals, resulting in weak reactivity of serological reagents. We tested neutralization of 7 tick-borne viruses in plaque-reduction tests with and without addition of accessory factor derived from normal guinea pigs. In nearly all cases the neutralizing capacity of the homologous antiserum was increased by 10- to 100-fold, while no instance of enhancement of cross reactivity was seen.

This technique was employed to retest certain sera previously shown reactive in tissue culture neutralization test (NT) with tick-borne viruse Results confirmed earlier findings: about 5% of 445 Alaskan males tested showed neutralizing antibodies to Poovoot virus. Sera collected in 1973 from common murres in Oregon were previously shown to be cross reactive in NT with the related viruses, Yaquina Head (from Oregon) and Poovoot (from Alaska). Use of the accessory factor greatly enhanced the avidity of the reaction with the sympatric virus, while having no effect on that with the Alaskan strain.

The DCA test (susceptibility of virus to sodium deoxycholate) is widely used to distinguish enveloped viruses from unenveloped ones and thus aid in identification and classification of field isolates. Certain tick-borne viruses (Orbiviruses) are resistant while the predominately mosquito-borne Togaviruses are susceptible to the action of DCA. We have observed a variable response of Kemerovo group viruses (Orbiviruses) to DCA treatment, which seems related to the period that virus is frozen. Stocks of Yaquina Head, Poovoot, Kenai, and Tribeč viruses stored frozen for a year or more show a marked susceptibility to DCA treatment while recently prepared, frozen stocks are markedly resistant. This indicates the need for caution in evaluating results of this important test.

Continuation of our studies on ticks as vectors of viral diseases is important because more of these viruses are being implicated in human disease. They are also important vectors of pathogens to domestic livestock. Our continued demonstration of the multiplicity of viruses and rickettsia in *Ixodes* ticks in North America is of potential epidemiological significance. Two recent findings by foreign workers attach additional importance to continuing our studies on ticks in the northwestern U.S. These are the isolation of Powassan virus from *Haemaphysalis* ticks by workers in Eastern Russia and the isolation of a virus related to Colorado tick fever from *I. ricinus* by workers in West Germany. The latter isolation is significant.

Keyword Descriptors: ticks, ixodid, argasid, viruses, disease relationships

Publications:

Hughes, L. E., Casper, E. A., and Clifford, C. M.: Persistence of Colorado tick fever virus in red blood cells. *Am. J. Trop. Med. Hyg.* 23: 530-532, 1974.

In press:

Yunker, C. E., Brennan, J. M., Hughes, L. E., Philip, C. B., Clifford, C. M., Peralta, P. H., and Vogel, J.: Isolation of viral and rickettsial agents from Panamanian Acarina. *J. Med. Entomol.*

Project No. Z01 AI 00081-14 RML
1. Rocky Mountain Laboratory
2. Epidemiology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The epidemiology of human infections of special interest

Previous Serial Number: NIAID-182

Principal Investigator: R. N. Philip

Other Investigators: R. L. Anacker, W. Burgdorfer, E. A. Casper,
R. A. Ormsbee, H. G. Stoenner, and L. A. Thomas

Cooperating Units: North Carolina Dept. of Human Resources, Division of
Health Services, Raleigh: Dr. D. J. Sexton and
Dr. J. N. MacCormack
Dr. M. Skinner, Montana Dept. of Health and
Environmental Sciences, Helena

Man Years:

Total:	3
Professional:	1 9/12
Other:	1 3/12

Project Description:

1. Colorado tick fever (CTF). During continued studies on the epidemiology of this disease in the Northwest we isolated CTF virus from 52 of 118 patients affected with tick-associated illnesses. During the first 3 days of illness virus was more readily obtained from serum than from clot. Thereafter, virus was erythrocyte associated.

Twenty-seven cases occurred in Ravalli County which is an astounding attack rate of 1.6 per 1,000 population, indicating the importance of this disease in this area. Of particular interest was the identification of a subclinical case. Virus was first identified in an attached tick which led to clinical follow-up of the patient. She remained well although she had viremia for at least 83 days.

In collaboration with Dr. Skinner, arrangements were made to test all sera obtained by the Red Cross in Montana between September 1974 and August 1975 for CTF-FA antibodies. Thus far, 3,100 sera from all areas of the state have been tested using CTF virus (Florio strain)-infected BHK cells as antigen. One percent of sera had low titers of CTF antibody, none of which exceeded 1:128. It is unlikely that any person had CTF during the preceding tick season. If transmission of CTF virus by blood transfusion occurs in Montana, it appears to be an unlikely event.

A direct FA test was devised to detect CTF antigen in the hemolymph and hemocytes of nymphal and adult Dermacentor andersoni ticks. The test is simple and reliable. No false positives were obtained when 100 experimentally infected ticks and appropriate controls were examined by FA and injected into suckling mice, and the procedure identified 80% of ticks that were virus-positive. The test should be particularly useful in early identification of CTF-virus infection in ticks removed from man. Whether or not one can predict the occurrence of disease in such instances remains to be determined.

2. Rickettsial infections. In collaboration with the Bureau of Communicable Disease Control, North Carolina Division of Health Services, we conducted a sero-epidemiologic study of Rocky Mountain spotted fever (RMsf) in North Carolina. All sera (total 546) submitted in 1974 for "rickettsial serology" were tested for spotted fever-group complement-fixing (CF) (Thomas) and immunofluorescence (micro-IF) antibodies. They are also being tested by passive hemagglutination (HA) (Anacker) and micro-agglutination (MA) (Burgdorfer). All sero-reactors, and some nonreactors, were followed up to determine clinical and epidemiologic characteristics of illness. This information has been computerized and will be correlated with serologic findings.

On basis of serology alone, 97 possible cases of RMsf were identified among the 347 patients from whom sera were obtained and tested. The fact that nearly 3 times as many patients with paired sera had diagnostic ($\geq$ 4-fold rise in titer) antibody responses by micro-IF as by CF is particularly important. Use of 10 CF units of antigen did not appreciably increase the sensitivity of the CF test.

If the specificity of the micro-IF test can be verified by correlation with clinical information, then this procedure may become the procedure of choice for routine serodiagnosis of RMsf. The test is simple and economical to perform and can readily be adapted to use in state health department laboratories. The antigen consists of yolk sac richly infected with rickettsiae, inactivated with formalin, and stabilized with merthiolate. Full reactivity is retained when held at 4° C for as long as 3 years, and 1.0 ml of antigen is sufficient for several thousand tests.

Antibodies begin to appear at about the 5th day of illness and reach maximum titers during the 3rd week. IgM antibodies are detected in 85% of cases and apparently persist for several months. Of particular interest to the epidemiologist is the fact that the same drop of diluted serum can be reacted against as many as 12 antigens simultaneously, thereby permitting study of antigenic differences in strains.

Although results are incomplete, the HA test appears to be even more sensitive than the micro-IF test. The significance of low titers has yet to be evaluated. The micro-agglutination test results are likewise incomplete, but the findings thus far correlate well with those by micro-IF. The HA and MA tests also detected some spotted fever antibody responses in CF test nonreactors.

We plan to continue serologic studies of RMsf in North Carolina. A particular goal this year will be to isolate rickettsiae from human cases for comparison with known spotted fever-group organisms in order to determine whether there are antigenic differences in infecting strains.

Keyword Descriptors: Colorado tick fever, Rocky Mountain spotted fever, tick-associated diseases, epidemiology, hemolymph test, immunofluorescence, complement-fixation, hemagglutination

Publications:

Philip, R. N., Casper, E. A., Gordon, F. B., and Quan, A. L.: Fluorescent antibody responses to chlamydial infection in patients with lymphogranuloma venereum and urethritis. J. Immunol. 112: 2126-2134, 1974.

Stoenner, H. G.: The biology of Borrelia hermsii in Kelly's medium. Appl. Microbiol. 28: 540-543, 1974.

In press:

Philip, R. N., Casper, E. A., Cory, J., and Whitlock, J.: The potential for transmission of arboviruses by blood transfusion with particular reference to Colorado tick fever. In Greenwalt, T. J. and Jamieson, G. A. (Eds.): Transmissible Disease and Blood Transfusion. New York, Grune and Stratton.

South, P. J. and Stoenner, H. G.: The control of outbreaks of leptospirosis in beef cattle by simultaneous vaccination and treatment with dihydrostreptomycin. Proc. 78th Annual Meeting of U.S. Animal Health Assn.

Project No. Z01 AI 00082-14 RML
1. Rocky Mountain Laboratory
2. Slow Viral Diseases
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Relation of viruses to the genesis of chronic disease

Previous Serial Number: NIAID-183

Principal Investigator: W. J. Hadlow

Other Investigators: M. E. Bloom, B. W. Chesebro, J. E. Coe, J. J. Munoz, J. L. Portis, and R. E. Race

Cooperating Units: Dr. C. M. Eklund, Hamilton, Montana (consultant)
Dr. A. L. Klingsporn, USDA Animal and Plant Inspection Service, Veterinary Services, Hyattsville, Md.
Dr. S. B. Prusiner, Dept. of Neurology, University of California, San Francisco

Man Years:

Total:	13
Professional:	2 7/12
Other:	10 5/12

Project Description:

From a study of naturally occurring slow viral disease of domestic animals, we hope to develop 1) an understanding of the mechanisms by which viruses cause protracted disease, and 2) working concepts and investigational procedures that will help determine whether viruses cause certain subacute and chronic diseases of man.

Clinicopathologic, virologic, and immunologic features receive most emphasis in this long-term study to obtain some understanding of the natural history and pathogenesis of scrapie and progressive pneumonia of sheep and Aleutian disease and transmissible encephalopathy of mink. Study of the causative viruses also receives attention but is of less immediate concern to this project.

Much of the year's effort was devoted to studies that ordinarily provide little reportable data in a 12-month period. Nevertheless, sufficient information accumulated on some aspects of the project do warrant comment here.

1. Scrapie and mink encephalopathy. In our continuing effort to understand the natural history of scrapie virus infection in Suffolk sheep, we examined 17 rams and ewes, all from high-risk pedigrees, that failed to become affected with the disease by the time they were 5 to 8 years old. By mouse inoculation, virus was not detected in 4 key non-neural tissues (ileum, upper colon, mesenteric node, and spleen). Whether these sheep had been infected earlier was not known. Nevertheless, our negative findings suggest that if virus had replicated earlier, it did not persist in the tissues examined. Thus, the question of whether early infection always betokens eventual disease is still unanswered. Observations on experimental scrapie virus infection in the goat strongly suggested that inapparent infection does not occur in this species. Factors that account for the occurrence of scrapie in only some sheep of a given high-risk pedigree remain to be identified.

The possible causal relation of scrapie virus infection to mink encephalopathy was strengthened by additional observations on the susceptibility of mink to "wild" scrapie virus from several sources. Thus, of 8 groups of mink inoculated intracerebrally with brain suspensions from different naturally infected Suffolk sheep, 7 have thus far become affected with a neurologic disease essentially like mink encephalopathy. Groups of mink similarly inoculated with brain suspensions from one Targhee sheep and from 2 goats likewise became affected. To date, only mink inoculated with brain suspensions from one Suffolk and one Cheviot sheep have not. Whether infected non-neural tissues, e.g., spleen and intestine, of the same donor sheep will also cause the disease is not known. Moreover, observations on the susceptibility of mink to scrapie virus introduced orally are needed to interpret the significance of our results vis-a-vis the most likely route of infection in the natural mink disease.

After an incubation period of about 20 months, mice inoculated with first passage mink brain became affected with a neurologic disease that somewhat resembled murine scrapie clinically, but whose pattern of neuropathologic changes was atypical. However, at least one mouse inoculated with second mink passage brain became affected with typical scrapie. Obviously, as observed by another investigator, one passage of "wild" scrapie virus in mink alters its ability to cause typical scrapie in the mouse. This phenomenon could account for the lack of pathogenicity of mink encephalopathy virus for mice if the natural mink disease represents nothing more than first passage of scrapie virus infection.

These observations on the susceptibility of mink to scrapie virus from sheep and goats are complemented by those on the occurrence of a disease indistinguishable from scrapie in sheep and goats inoculated intracerebrally with mink encephalopathy virus. During the past year, another Cheviot sheep became affected (almost 7 years after inoculation) to bring the total to 6 of 19 as compared with 10 of 19 goats. A second passage in goats is in progress to determine the effect this might have on pathogenicity of the virus for mink and mice.

2. Aleutian disease (AD). The likelihood of different strains of AD virus in nature and the possible bearing this may have on epidemiologic features of the disease and on results of experimental studies have received little attention. Our recent studies have confirmed the existence of several strains that differ dramatically in their pathogenicity for mink of non-Aleutian genotype, the basis we have used for assessing strain differences. Four strains (Utah-1, Pullman, Ontario (Canada), and Bitterroot) are under study. Results obtained so far place Utah-1 as the most virulent and Pullman the least virulent in non-Aleutian (pastel) mink. The Bitterroot strain falls in between. The Ontario strain probably resembles Utah-1 more closely than the other 2. All strains seem equally pathogenic for mink homozygous for the Aleutian gene (e.g., sapphire color phase).

Detailed comparative studies of these strains in animals would be time-consuming and expensive, but they would provide data on the kinetics of virus replication that are essential to any critical comparison. We know the patterns of virus replication of Utah-1 and Pullman strains differ; peak titers of virus in key tissues occur much earlier with the Utah-1 strain. This has a bearing on their usefulness in the preparation of antigen for the counter immunoelectrophoresis test. When tissues for antigen were harvested 9 or 10 days after mink were inoculated intraperitoneally, infection with the Utah-1 strain provided satisfactory antigen whereas the Pullman strain did not. Although these several strains differ in their biologic behavior, we do not know whether antigenic differences exist among them. Antibody to all is readily detectable by the counter immunoelectrophoresis test in which purified Utah-1 virus is used as antigen.

To better define the differences in response between Aleutian and non-Aleutian mink inoculated with the Pullman strain of AD virus, we sought more data on the temporal features of its replication in pastel (non-Aleutian) mink. Limited observations confirmed the occurrence of great individual variation in response within groups of 3 mink examined for virus at sequential intervals after inoculation. This was reflected not only in the tissue distribution of virus but also in the titers reached in individual tissues at any given interval. As was surmised from earlier studies, replication of virus occurred much more readily in some mink than in others even though overt disease does not occur in most pastels that receive moderate amounts of the Pullman strain. Specific antibody, however, appeared in all of them.

Pastel mink that did not become affected with AD but did develop specific antibody after inoculation with Pullman strain virus were not protected when challenged with graded amounts of Utah-1 strain virus. AD occurred in all of them, sometimes after a much shortened incubation period. These findings, and similar ones reported last year, need to be examined more completely, but at present they suggest that mink with specific antibody may be more susceptible to virulent AD virus. For this reason, use of a vaccine to control the disease may not be feasible.

3. Progressive pneumonia. Nothing new can be reported on the 2 small groups of sheep and goats inoculated with progressive pneumonia virus.

The possible role of viruses in the genesis of chronic disease in man continues to attract attention of biomedical investigators. Because of the complex problem, diverse approaches are used to gain information that will provide a clearer understanding of possible mechanisms involved. One of the more fruitful, though apparently less fashionable, approaches has been the study of certain naturally occurring diseases of domestic animals. It has provided insights into the problem that could not have been gained in any other way. Indeed, our observations not only formed the basis for the original concept of the causal relation of viruses to chronic disease, but also provided critical clues that led to the demonstration that at least two protracted neurologic diseases of man are caused by viruses. For these reasons, this project offers a promising new horizon for the Institute in its abiding concern for infectious disease.

Loss of 2 more persons from the Slow Viral Diseases Section (one professional member and one animal caretaker) has forced further reduction in the total effort of the project. Thus, studies on progressive pneumonia will be discontinued and those on mink encephalopathy will be curtailed. Existing work on scrapie will continue as a few more young sheep from the Mission, Texas, flock are examined. The greatest effort will be devoted to Aleutian disease, which has attracted more collaborative interest within the laboratory.

The loss of momentum in the existing project during the past 5 years and our inability to initiate complementary studies with human material are regrettable, especially in view of the laboratory's need for a sustaining research theme. Despite this, a continued effort will be made to work toward the objectives of the project while it is still viable.

Keyword Descriptors: chronic human disease, slow viral disease, scrapie, mink encephalopathy, Aleutian disease, progressive pneumonia, domestic animals, sheep and goats, mink

Publications:

Hadlow, W. J., Eklund, C. M., Kennedy, R. C., Jackson, T. A., Whitford, H. W., and Boyle, C. C.: Course of experimental scrapie virus infection in the goat. J. Infect. Dis. 129: 559-567, 1974.

Hadlow, W. J.: Myopathies of animals - a brief view. In Homburger, F. and Lucas, I. (Eds.): Comparative Pathology of the Heart. Advances in Cardiology. Basel, S. Karger, 1974, vol. 13, pp. 68-80.

Cork, L. C., Hadlow, W. J., Gorham, J. R., Piper, R. C., and Crawford, T. B.: Pathology of viral leukoencephalomyelitis of goats. Acta Neuropathol. 29: 281-292, 1974.

Project No. Z01 AI 00083-07 RML
1. Rocky Mountain Laboratory
2. Medical Zoology and Zoonotic Diseases
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Host-ectoparasite relationships

Previous Serial Number: NIAID-187

Principal Investigator: J. F. Bell

Other Investigators: None

Cooperating Units: Dr. W. A. Nelson, Research Station, Canada Department of Agriculture, Lethbridge

Man Years:

Total:	11/12
Professional:	1/12
Other:	10/12

Project Description:

Inquiry into the nature of acquired and innate resistance of vertebrates to ectoparasites has been continued, emphasizing histopathology of the skin of naive and sensitized rabbits infested with ticks, at various stages of infestation, and histopathology of mice of the C_{57}/bl (innately susceptible to lice) and CFW (innately resistant) breeds at various stages of infestation. The purpose of these studies is to obtain a clue as to the reactions that inhibit the feeding of ectoparasites with resultant decrease in their fertility and viability. Mice tolerant to lice are being studied comparatively to aid in identifying the resistance mechanism.

Tolerance to lice could not be induced by inoculation of louse extracts into neonates and by intermittent feeding of lice on mice from birth. Subsequent attempts by continuous exposure during infancy also failed to induce tolerance. The phenomenon seems to be innate rather than acquired.

The relationship of the immune phenomena of ectoparasitism to resistance is not much advanced beyond the observations of Trager (1939) although immunoallergic reactions of several kinds are prominent. The same can be said of dermatophytoses and I (J.F.B.) believe the resistance reactions to the two kinds of infestation may be related.

Keyword Descriptors: ectoparasite, resistance, skin, histopathology, strain, ticks, lice, keds

Publications:

In press:

Nelson, W. A., Keirans, J. E., Bell, J. F., and Clifford, C. M.: Host-ectoparasite relationships. J. Med. Entomol.

Project No. Z01 AI 00084-06 RML
1. Rocky Mountain Laboratory
2. Medical Zoology and Zoonotic Diseases
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Vector-pathogen relationships

Previous Serial Number: NIAID-188

Principal Investigator: C. E. Yunker

Other Investigators: U.K.M. Bhat (NIH Visiting Fellow), C. M. Clifford, J. E. Keirans, R. A. Ormsbee, and L. A. Thomas

Cooperating Units: Dr. Kouka S.E. Abdel-Wahab, Al Azhar University, Cairo, A.R.E.
Dr. H. Hirumi, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York
Dr. J. A. Howarth, University of California, Davis
Dr. M. N. Kaiser, NAMRU-3, Cairo, A.R.E.
Dr. C. W. Ng, Laboratory Centre for Disease Control, Canada Dept. Health and Welfare, Ottawa, Ontario

Man Years:

Total:	2 9/12
Professional:	1 8/12
Other:	1 1/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
PL-480 03-016-N	C. E. Yunker	NAMRU-3 Cairo, A.R.E.	M. N. Kaiser	$14,000
PL-480 03-030-N	C. E. Yunker	Al Azhar Univ. Cairo, A.R.E.	K.S.E. Abdel-Wahab	0

Project Description:

The objective of this project is to investigate the host-range, cellular relationships, and growth dynamics in bloodsucking arthropods or their cells of pathogenic agents transmissible by vectors among man or animals.

The virus-plaquing ability of 7 lines of mosquito cells was tested in comparison with Singh's Aedes albopictus cell line. The Aedes cells all withstood 37° C; Ae. albopictus cells plaqued 10 of 11 viruses, Ae. w-albus plaqued 4, and Peleg's Ae. aegypti plaqued 3 viruses. The remainder (Singh's Ae. aegypti, Varma and Pudney's Ae. aegypti, and 2 lines from Culex spp.) failed to form plaques with any virus. Both Singh's Ae. w-albus and Peleg's Ae. aegypti cells gave better yields of vesicular stomatitis (Indiana) virus than did Ae. albopictus cells.

Recently we demonstrated that dengue viruses will readily plaque in the Ae. albopictus cell line, which is known to be a superior system for the isolation of these viruses based on cytopathogenic effect. We then compared dengue virus-induced CPE with plaques in these cells and in Vero cells under various conditions. The study, still in progress, reveals that dengue viruses 1 and 3 will induce syncytia formation in mosquito cells only at very high concentrations, indicative of "fusion from without". With these 2 viruses, better development of syncytia is seen at 37° C than at 27° C. Dengue-4 virus causes little or no syncytia, whereas dengue-2 readily causes syncytia in mosquito cells regardless of temperature of incubation. However, dengue-2 immune sera fails to inhibit homologous syncytia formation. Dengue-2 plaque titers are consistently higher in Ae. albopictus than in Vero cells receiving identical inocula. The various dengue viruses were not separable by cross plaque-reduction neutralization tests in mosquito cells.

Because virus production in mosquito cell monolayers is limited to only a small proportion of the cell population, it was thought that clonal populations might have differing susceptibilities to virus infection. Ten clones of Ae. albopictus cells were compared with regard to morphology, growth characteristics and virus-supporting ability. Nine arboviruses known to plaque in the parental strain of cells and 2 that do not were tested for plaquing in all cell populations. Some selectivity was seen, but with most viruses the parent cell line was most efficient. In 8 tests, a mosquito-borne virus, Chikungunya, which plaques inconsistently in the parental strain of cells, repeatedly plaqued in 2 of the clonal populations. This ability was correlated with cell morphology and growth characteristics of the cloned cells. The superiority of the Ae. albopictus cell line for the primary isolation of certain viruses of low pathogenicity apparently may be improved by cloning.

In 1973 we isolated a syncytium-inducing agent from a substrain of Singh's Ae. albopictus cells maintained by another laboratory. The agent was identified as a togavirus of group A, serologically closely related to Chikungunya virus. Strains of Ae. albopictus cells kept at various laboratories were examined for contamination with this virus. Electron microscopy of syncytia in 2 substrains of Singh's Ae. albopictus cells revealed the presence of 5 different types of virus-like particles, which morphologically resembled parvo-, toga-, orbi-, picorna, and bacterial viruses. When a virus-free substrain of this cell line was inoculated with extracted syncytia the first 3 types of agents were consistently observed. Among these agents, the togavirus-type appears to be responsible for syncytium induction. The Ae. albopictus cell line has recently become important in the study of arboviruses, especially the dengues, which also cause syncytia formation. The existence of undetected or latent viruses in these cell cultures could present a serious complication to diagnostic and experimental studies of arboviruses, as well as a hazard to those who work with these cells.

A rickettsia-like agent from bloods of a chipmunk, Eutamias ruficaudus, obtained by plaquing in mosquito and Vero cells failed to yield a usable CF antigen when grown in yolk-sac of embryonated eggs. However, antigens prepared from infected Vero cell cultures reacted specifically with sera against strains of Wolbachia sp. from tick sources, as well as with homologous antiserum. Further work on the identity and characterization of this agent is in progress.

It has been noted that, while tissue and cell cultures have been used for the propagation of various rickettsiae, the value of such systems in primary isolation and identification has not been confirmed. Our results indicate that cell cultures are applicable to isolation and identification of rickettsial and rickettsia-like organisms that are not readily studied in standard egg or animal systems.

Although much effort has been made in the past to separate rickettsiae from host-tissue components, apparently little attention has been paid to species purity of standard strains. We used serial plaque purification techniques to "clone" populations of 8 rickettsial strains currently employed as standard test organisms. These will be used to prepare antigens and antisera, which can be compared with those of the parental strain for avidity and cross reactivity in serological tests.

Efforts were renewed to establish a line of tick cells. Two hundred eighty-six primary cultures were prepared from 8 species of Ixodidae, mostly Dermacentor andersoni. Emphasis was placed on methodology successfully employed by British workers, who recently established the first permanent line of cells from a tick, Rhipicephalus appendiculatus, and on those found successful for mosquito cell culture. Results were no more encouraging than our previous ones in which varied methodology was used. In contrast, a single attempt to establish a permanent line of cells from the yellow-fever mosquito, Aedes aegypti, succeeded, demonstrating the ease with which mosquito cell lines, as opposed to those of ticks, may be established. Future efforts will explore the premise that certain tick species may be more easily established in culture than others, especially D. andersoni.

In the search for growth stimulants for arthropod cells in vitro, growth of our cloned, serum-free substrain of Ae. albopictus cells was assayed in medium containing 4 known growth-enhancing factors or combinations thereof. Significant improvement in growth was seen only when 0.5% bacto-peptone was added to lactalbumin-hydrolysate medium containing 1% bovine plasma albumin. Tryptose phosphate broth was shown to enhance growth of the serum-dependent parental strain of Ae. albopictus cells and inhibited CPE caused by 3 viruses. The significance of this finding for our studies will be investigated.

Progress of PL-480 agreement 03-016-N (Tick biochemistry and physiology) has proceeded along the lines agreed to during an on-site visit by the Project Officer. Eleven research papers were published during the year, 4 are in press and 6 more are in various stages of preparation. Topics include: lipids, nitrogenous excretion cycle, spiracular function, diapause, new fatty acid, sterol metabolism, water balance and starvation, juvenile hormone, bloodmeal breakdown, and haem synthesis.

We continued to supply, upon request, training, cell cultures, and information to RML personnel and visitors, as well as to outside institutions. In support of projects AI 00079-16 RML and AI 00081-14 RML we prepared numerous California group and Colorado tick fever virus antigens in cell cultures. Also, for the latter project, we assayed growth and CPE in various cell cultures of an unidentified virus isolated from ticks collected in the Tennessee Valley and performed various plaque reduction neutralization tests.

Keyword Descriptors: arboviruses, rickettsiae, vector cells, tissue culture, ticks, mosquitoes, biochemistry, physiology

Publications:

Cory, J., Yunker, C. E., Ormsbee, R. A., Peacock, M., Meibos, H., and Tallent, G.: Plaque assay of rickettsiae in a mammalian cell line. Appl. Microbiol. 27: 1157-1161, 1974.

Yunker, C. E. and Cory, J.: Plaque production by arboviruses in Singh's *Aedes albopictus* cells. *Appl. Microbiol*. 29: 81-89, 1975.

Cory, J. and Yunker, C. E.: Rickettsial plaques in mosquito cell monolayers. *Acta Virol*. 18: 512-513, 1974.

In press:

Cory, J., Yunker, C. E., Howarth, J. A., Hokama, Y., Hughes, L. E., Thomas, L. A., and Clifford, C. M.: Isolation of spotted fever group organisms and *Wolbachia* sp. from field-collected materials by means of plaque formation in mammalian and mosquito cells. *Acta Virol*.

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